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Effect of Low Dosage Fluorides on Bacterial Metabolism, Growth and Attachment

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THE EFFECT OF LOW DOSAGE FLUORIDES ON BACTERIAL METABOLISM, GROWTH AND ATTACHMENT

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Review of the Literature

That dental caries results from dissolution of enamel by acid produced by bacteria adherent to teeth now seems well established (for review, see Fitzgerald, 1976). Furthermore, the anti-caries effect of fluorides was believed for many years to be due solely to the physicochemical conversion of hydroxyapatite to less acid soluble fluorapatite (for reviews, see Gray et al., 1962; Brudevold and Soremark, 1967). The change in enamel solubility hypothesis is, however, no longer believed able to explain all of the caries inhibition role of fluoride. Rats exposed to low levels of fluoride in drinking water have shown marked caries reduction without a change in enamel fluoride levels (Larson et al., 1976). In addition, the fluoride content of exfoliated primary teeth does not correlate with a child's caries experience (Englander and Mellberg, 1976); while enamel fluoride levels determined for enamel biopsy specimens, on an individual basis, has no relationship to past caries experience (Poulsen and Joost Larsen, 1975; DePaola et al., 1975), nor as a predictor of new caries attack (Shern et al., 1977; Parkins et al., 1975).

The clearly demonstrated caries reducing effect of fluoride in the absence of chemical changes in the fluoride content of enamel has spurred investigation of the possible role of the antimicrobial action of fluoride in caries reduction. Many studies have been performed to examine potential antiplaque effects of fluoride, but most of these studies have only used the compound sodium fluoride as a test agent.

This review will address several topics which suggest that fluoride affects the bacteria in plaque and that specific fluoride compounds may be more effective than others. Particularly, studies will be cited which

demonstrate that fluoride can alter bacterial acid production, glucose uptake, and cell membrane transport of metabolites. Hypotheses will be presented that suggest how fluoride might reduce bacterial accumulation on teeth through altered polysaccharide production, interactions with heavy metals, competition for binding sites, or change of enamel surface energy. Studies which have demonstrated bacteriostatic and bactericidal effects by relatively high levels of fluoride will also be presented. Finally, recent research which has noted specific fluoride compounds that clinically have a marked antiplaque effect will be reviewed.

Fluoride Inhibition of Microbial Carbohydrate Metabolism

Bibby and Van Kesteren (1940) were the first to show that glycolysis by pure cultures of oral streptococci was inhibited by concentrations of fluoride as low as 1 ppm. Wright and Jenkins (1954) confirmed this finding when they showed that acid production by mixed microbes in saliva-glucose mixtures was inhibited by concentrations of 1 ppm F. Jenkins (1959) furthermore demonstrated with mixed cultures of oral bacteria that the degree of fluoride inhibition of carbohydrate metabolism was increased by decreasing the pH of the medium. This was subsequently confirmed in studies with pure cultures of oral streptococci (Weiss et al., 1965; Hamilton, 1969b).

In addition to inhibition of glycolysis, the addition of low levels of fluoride results in the inhibition of glycogen synthesis by oral bacteria (Sandham and Kleinberg, 1969b; Weiss et al., 1965; Hamilton, 1969b). This has been speculated to be due to interference with glucose transport across the cell membrane by fluoride (Weiss et al., 1965; Hamilton, 1969a, b; Sandham and Kleinberg, 1969b). Recently Miller (1974, 1976) showed that a decrease in S. faecalis acid production could

be obtained at 0.5 ppm F, yet inhibition of glucose transport was not demonstrated until at least 10 ppm F was used. These results suggest the possibility of a dual but separate inhibitory action of fluoride on both the microbial glycolytic and glucose transport mechanisms. Inhibition of glucose transport by fluoride is of importance since significant numbers of organisms in dental plaque (Gibbons and Socransky, 1962) and salivary sediment (Sandham and Kleinberg, 1969a) have been shown to form large quantities of intracellular polysaccharide. These glycogen stores have been implicated in prolonging enamel exposure to acid (Gibbons and Socransky, 1962).

Fluoride and Cell Membrane Transport

The recent work of Luoma (1972, 1973) and Luoma and Tuompo (1975) with cariogenic streptococci suggest that fluoride functions by decreasing the cellular potassium, and to a lesser degree, by decreasing the cellular phosphate content accompanied by an initial rapid uptake of fluoride into the cells. This change in ionic transport activity was noted to be greater at lower pH values. Luoma and Tuompo (1975) found that a close association exists between potassium transport and carbohydrate metabolism, suggesting that these two processes probably occur through a related mechanism within the cell membrane.

Fluoride Reduction of Bacterial Plaque

The ability of various oral streptococci, particularly S. mutans, to synthesize extracellular glucans during the metabolism of sucrose has been thought to be a significant factor in their colonization of the tooth surface and in the dental caries process (for review, see Gibbons and van Houte, 1973). Because of its importance in microbial aggregation and plaque formation, particular interest has been shown in the

synthesis of extracellular glucans (Gibbons and van Houte, 1973; Gibbons and Fitzgerald, 1969). A fluoride level of 10 ppm has been noted to produce a reduction in extracellular polysaccharide synthesis by bacteria in subjects receiving fluoride-sucrose mouthrinses (Loesche et al., 1973 and 1975). These results may be due to specific inhibition of S. mutans, an organism known to synthesize large quantities of extracellular polysaccharide (Gibbons and van Houte, 1973; Gibbons and Fitzgerald, 1969). Bowen and Hewitt (1974) also have demonstrated that 70 ppm fluoride increased the fructose-glucose ratio in the extracellular polysaccharides (EPS) of four strains of S. mutans; and Shimura and Onisi (1978) observed an overall decrease in alkali soluble glucan when S. mutans Ingbritt and ACTC 10556 strains were exposed to 15 ppm F for up to 48 hours.

Recently, Treasure and Handelman (1980) verbally reported that EPS/bacterial mass ratios of several strains of S. mutans vary in the presence of 25 to 50 ppm F. In contrast to previous findings, they found fluoride increased EPS synthesis. These results reported as EPS/mg bacteria ratios instead of the total EPS per unit volume of culture may more properly assess the influence of fluoride on bacterial EPS production.

Heavy Metal-Fluoride Interactions with S. mutans

Some fluoride compounds, i.e., stannous fluoride, have a relatively heavy cationic moiety; but the available knowledge of heavy metal interactions with bacteria is limited and scattered (Jernelöv and Martin, 1975). The toxicity of various metals on oral bacteria has been evaluated to some extent by Beighton and McDougall (1978). However, there is essentially no information on tin reactions with bacteria and on transport of metal ions through cell membranes.

Recently, Tinanoff and Camosci (1980a) have reported electron dense

granules present in and on S. mutans treated with SnF_2. Transmission electron microprobe analysis demonstrated these granules to have x-ray energies consistent with tin. Review of stained and unstained specimens from previous experiments that were treated intermittantly with SnF_2 revealed these same electron dense granules. $SnCl_2$ treated cells, however, did not exhibit these granules. Subsequent tin analysis of bacterial samples exposed to SnF_2 or $SnCl_2$ showed the amount of tin per mg plaque was 11 times greater in the SnF_2 group than that found in the $SnCl_2$ group. These results suggested that when bacterial cells are exposed to SnF_2, tin enters the cells in large quantities and its transport into the cell was enhanced in the presence of fluoride ions. The differences in bacterial cell uptake of fluoride and chloride ions may partially explain the differences of tin deposition in plaque treated with SnF_2 and $SnCl_2$. Fluoride accumulates within the plaque (Jenkins and Edgar, 1969; Whitford et al., 1977), whereas chloride apparently does not (Shultz et al., 1962; Mitchell and Moyle, 1959). Hence, the large accumulation of tin found in the bacterial cells exposed to SnF_2 could possibly be explained by the transport and accumulation of fluoride in the cells, with tin passively entering the cells. This granule formation is not unlike the response of other cells to heavy metals and is believed to be a method by which cells "detoxify" foreign elements (Simkiss, 1977).

Alteration of Binding Sites

In vitro studies have demonstrated that fluoride may alter the adherence of S. mutans to the tooth surface. Clark et al. (1973) demonstrated that pretreatment of hydroxyapatite by fluoride could reduce the adherence of sucrose grown S. mutans to hydroxyapatite.

According to Bernardi and Kawasaki (1968) and Bernardi et al., (1972) hydroxyapatite may be regarded as an amphoteric substance which may bind both positively and negatively charged molecules. This binding may be competitively inhibited by free negatively charged ions which have a well established high affinity for calcium ions. Since fluoride and phosphate ions have a well established high affinity for calcium ions, these agents may act to inhibit or desorb salivary glycoproteins and bacteria from the enamel surfaces.

Furthermore, Rölla (1976) found that fluoride inhibited the attachment of bacteria to each other as well as to hydroxyapatite, whereas calcium ions increased the attachment. Kelstrup and Funder-Nielsen (1972) and Hay et al. (1971) showed that calcium was essential for the action of agglutinating factors in plaque, presumably because calcium forms bridges between negatively charged bacterial surfaces. It has been suggested that phosphate and fluoride ions might interfere with these calcium bridges (Rölla, 1976) and in turn, sufficiently alter the bacterial attachment to produce the cariostatic effect of these agents in vivo.

Recently, there has been interest in highly charged phosphorylated compounds which may be involved in bacterial adhesion to enamel. A negatively charged macromolecule produced by bacteria known as teichoic acid seems to form complexes with cell surface bacterial polysaccharides, possibly increasing the affinity of these polysaccharides for tooth surfaces (Markham et al., 1975). One of the suggested roles of teichoic acid is that of a mediation of bacterial attachment to enamel surfaces (Rölla, 1975; Svatun et al., 1977). In a study using stannous fluoride, Svatun et al. (1977) concluded that a negatively charged ion such as fluoride may compete with teichoic acid for binding sites on the enamel surface by forming complexes with calcium ions. Also, tin a heavy

metal ion, may bind with negatively charged groups on the bacterial surface possibly decreasing bacterial attachment. Stannous fluoride may, therefore, have a double effect on bacterial binding sites.

Alteration of Enamel Surface Energy

Exposure of dentin or enamel to aqueous stannous fluoride may decrease the ability of plaque to adhere to the treated tooth surfaces due to a decrease in surface energy (Glantz, 1969) as measured by contact angle. Brittain et al. (1974) in a study of the effect of fluoride on adherence of S. mutans to tooth enamel in vitro, found that both stannous fluoride and acidulated phosphate fluoride/stannous fluoride treatments were effective in reducing the number of microorganisms on the enamel surface. Caldwell et al. (1977) also found in an in vivo study that the area and the amount of plaque formed on the stannous fluoride treated teeth were significantly less than that on untreated control teeth. These authors attributed the antiplaque effect of stannous fluoride to an alteration of enamel surface energy which interfered with bacterial attachment. However, the possibility of other mechanisms was not ruled out.

Bacteriostatic and Bactericidal Effects of Fluoride

Bacteriostatic and bactericidal effects of fluoride have been shown in studies using relatively high concentrations of fluoride over long periods of time. Bacterial growth rates were shown to be reduced in vitro by concentrations of sodium fluoride varying from 50 to 200 ppm (Shiota, 1956; Bowen and Hewitt, 1974; Miller, 1974; Miller et al., 1976; Keene, 1974). Also, Keene et al. (1976) reported a decrease of S. mutans in human dental plaque with the use of 25,000 ppm stannous fluoride and Loesche et al. (1973 and 1975) suggested a reduction in

S. mutans in vivo using 12,000 ppm acidulated phosphate fluoride (NaF in 3.2 pH phosphate buffer). Depending on the concentration of fluoride used, the effects on bacterial growth ranged from bacteriostatic to bactericidal. Loesche (1977) suggested that high concentrations of fluoride affect bacterial growth thereby altering the proportions of bacteria found in dental plaque.

Action of Specific Fluoride Compounds

Various fluoride compounds including stannous fluoride have been tested clinically for plaque inhibiting effects. Frequent topical applications of stannous fluoride in rats greatly reduced bacterial accumulation on teeth (König, 1959). Andres et al. (1974) reported a 99 percent reduction in salivary bacteria with stannous fluoride at 1250 ppm while sodium fluoride and saline controls failed to show this effect. In an in vivo study, Tinanoff et al. (1976c) found that stannous fluoride had a greater effect on plaque than sodium fluoride. When the stannous fluoride mouthrinse was used once or twice daily for a two day period, the bacterial colonization was greatly reduced. Since the bacteria in the SnF_2 treated plaque ultrastructurally showed decreased adhesion and cohesion, these effects were suggested to be the result of alteration of attachment properties between enamel and bacteria and between bacteria and bacteria.

Rölla (1976), commenting on these results, suggested that the increased suppression of bacterial colonization of enamel by stannous fluoride may be due to the effects of tin ions. However, in a few samples where stannous chloride was used as a mouthrinse (Tinanoff, 1976c), no dramatic reduction in plaque was found. In a more recent experiment, stannous chloride equimolar to SnF_2 was noted to have some plaque reduction but was not nearly as effective as that found with SnF_2

(Skjörland et al., 1978). Even though this area needs more investigation, the variation in microbial colonization on enamel may not be due to the tin ion alone.

An in vivo microbiologic investigation of stannous fluoride mouthrinse using a similar methodology to that used by Tinanoff in 1976, revealed that this agent was associated with a large decrease in the number of bacteria attached to enamel (Gross and Tinanoff, 1977). Following a two day period when stannous fluoride was used, the number of bacteria attached to enamel was reduced by 96 percent. Further in vivo studies have shown that several fluoride compounds tested for antiplaque properties may affect bacterial accumulations on teeth (Tinanoff et al., 1978). Although sodium fluoride, acidulated phosphate fluoride and sodium monofluorophosphate mouthrinses did not appear to affect plaque formation, reduction was noted with stannous fluoride, sodium hexafluorostannate and two amine fluoride mouthrinses.

The clinical effects of frequent mouthrinses with stannous fluoride have recently been reported in experimental animals and in man. Shern and Couet (1977) found stannous fluoride significantly more effective in reducing plaque in rats than an agent coded as DAPA-1. The groups receiving stannous fluoride and DAPA-1 had 71 percent and 12 percent less plaque respectively than the controls. Svatun et al. (1977) found that stannous fluoride at 0.3% (750 ppm F^-) was as effective as 0.1% chlorhexidine in reducing plaque. In a recent report, Yankell et al. (1978a) using two groups of human subjects who did not brush but rinsed twice a day with either 250 or 1,000 ppm stannous fluoride, found plaque reduced 74-84% and 90-99% respectively. Results in a second study showed that all stannous fluoride plaque indices were significantly lower than control values (Yankell et al., 1978b). Tinanoff et al. (1980c) has

further confirmed the efficacy of SnF_2 as an antiplaque agent in another clinical study using a SnF_2 (250 ppm F) mouthrinse twice daily on 27 dental students for 5 days. Significant reductions in plaque score, plaque wet weight and the number of viable bacteria on teeth were observed in the SnF_2 group.

Conclusions

From this review certain points should be emphasized. The fluoride ion affects not only enamel solubility as previously thought, but may also influence caries activity and plaque formation by altering bacterial growth and attachment. To date, most of the published studies have used NaF to evaluate the fluoride ion effect on bacterial plaque. Few studies have compared different fluoride compounds despite the fact that SnF_2 appears to be more effective in this regard. There have been only scattered studies or reviews on alteration of bacterial growth, acid production, polysaccharide production and attachment by various fluoride compounds. Some of these studies have been performed at sufficiently high concentrations of F that either a bactericidal or bacteriostatic effect of the test agent is a factor. It is thus of value to test the antiplaque properties of low levels of various fluoride compounds to determine if differences exist in their antiplaque mechanisms.

Hypotheses

Because the physicochemical interaction of fluorides with enamel cannot explain all the caries reductive effect of this ion clinically, the experiments in this thesis will test the effect of fluoride compounds on S. mutans NCTC 10449S, a bacteria known to be associated with dental caries. The literature suggests that different fluoride compounds affect bacterial growth and viability to different degrees. It would, therefore, be reasonable to assume that these compounds at low concentrations may also affect bacterial extracellular polysaccharide production, bacterial attachment, bacterial acid production and bacterial DNA synthesis. A final hypothesis would also suggest that the heavy metal moiety associated with certain fluoride compounds, as well, synergistically affect all of these parameters.

Specific Objectives

The experiments of this study were designed to determine the effect of continued exposure of low concentrations of NaF, SnF_2, Na_2SnF_6, TiF_4, and $SnCl_2$ on plaque formation by Streptococcus mutans NCTC 10449S. The specific objectives were therefore:

1) to determine the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of the test agents on S. mutans NCTC 10449S.

2) to determine the effect of continual exposure of the test agents at low concentrations on growth and acid production on S. mutans NCTC 10449S and to assess the individual or combined fluoride ion, cation or pH effects of these test agents.

3) to determine the quantity of tin present in bacterial plaque after exposure to the appropriate agents through atomic absorption

spectrophotometry.

4) to evaluate the quantity of bacteria through DNA analysis, attached to the test enamel and left unattached after exposure during growth to the various test agents or appropriate controls.

5) to evaluate the quantity of alkali and water soluble extracellular polysaccharide through glucan analysis and glucan/DNA ratios of the bacteria attached to enamel and of the unattached bacteria after exposure during growth to the various test agents or appropriate controls.

6) to qualitatively evaluate the effects of the various test agents through electron micrographic observation of bacterial ultrastructure in plaque.

Materials and Methods

Enamel Specimen Preparation

Enamel sections approximately 180 mm^2 were cut from the smooth surfaces of bovine incisors using a diamond drill with water coolant. A hole was placed in each specimen so that .030" stainless steel wires could be used subsequently to suspend the enamel in a test tube (Figure 1). The enamel specimens were cleaned with a slurry of pumice to remove organic material, washed with deionized water in a ultrasonic cleaner, and then autoclaved. Wax (inlay casting wax, Kerr products, Emeryville, CO) was used to cover the cut inner aspects of tooth leaving only the surface enamel exposed. The specimens were placed in 70% ethyl alcohol for 15 minutes to disinfect the surfaces from microbial contamination during wax preparation and then placed in sterile deionized water for 10 minutes to remove residual alcohol. Care was taken to assure that the enamel cylinders were not dessicated during preparation.

Microorganisms, Media, and Growth

A streptomycin resistant mutant, *Streptococcus mutans* NCTC 10449S (Bratthall serotype *c*; Tanzer et al., 1976), was selected as the test organism since this organism has been noted to 1) attach to enamel in a similar way as organisms *in vivo* (Tinanoff et al., 1978); 2) causes caries (Tanzer et al., 1976; Tanzer, 1979); and 3) is the most frequently found serotype in human population (Bratthall, 1972; Keene et al., 1977). Stock cultures were maintained by monthly transfer in fluid thioglycolate medium (Difco) supplemented with meat extract (20% $^{V}/V$) and excess $CaCO_3$. For MIC/MLC determinations, stock cultures were adapted for growth in trypticase soy basal medium (TSB; BBL). For all other experiments, stock cultures

Figure 1: Example of suspended bovine enamel section and adherent microorganisms after three days response to test agents or control in Jordan's medium with 5% sucrose. Organisms attached to the wire were used for tin analyses. DNA and glucan analysis were performed on organisms attached to the enamel, and on unattached organisms in the growth media.

were adapted for growth in Jordan's medium (Jordan et al., 1960) supplemented with 5% sucrose and 50 mg Na_2CO_3 (Figure 2). All experiments were performed at 37°C under microaerophillic conditions.

Fluoride Compounds and Controls

Fresh sterile solutions of the appropriate fluoride compounds or suitable control solutions were added to the sterile test medium to obtain the proper final fluoride or control dilution prior to inoculation of microorganisms. To obtain the appropriate dilutions, stock solutions of fluoride compounds were first prepared at 100 ppm F. For DNA/glucan testing, these stock aqueous solutions NaF (0.022% $^{w}/v$, pH 5.3), SnF_2 (0.041%, pH 3.8), Na_2SnF_6 (0.024%, pH 3.5), and TiF_4 (0.016%, pH 2.9) were added to Jordan's medium supplemented with 5% sucrose to produce fluoride concentrations of 10 ppm or 5 ppm F. Stock solution of $SnCl_2$ (0.05%, pH 2.9), which was equimolar to Sn in SnF_2 100 ppm F, was prepared and subsequently diluted into Jordan's medium, served as a control for tin. Deionized water (pH 6.6) was added to the Jordan's medium at the same volume as the other aqueous solutions and this medium served as the control to which all solutions were compared. The final pH values in all cases was 7.6.

For MIC/MLC testing, dilutions of the test agents to the final concentrations in trypticase soy basal medium necessitated higher concentrations of stock test solutions to be made. Solutions of NaF, ranging from 50 to 5000 ppm F; SnF_2, from 0.5 to 250 ppm F; Na_2SnF_6 and TiF_4, from 100 to 1000 ppm F; and $SnCl_2$, from 50 to 1000 ppm Cl were prepared (for example, see Figure 3).

Organic Binding of Free Fluoride

To rule out possible organic binding and to insure the accuracy of theoretical fluoride levels of this ion in the complex medium, free fluoride was determined by analysis with a Orion 90-09A fluoride electrode (Orion Research Laboratories, Cambridge, MA) connected to a digital electrometer. One ml samples of uninoculated and inoculated test media were evaluated immediately after addition of the fluoride agent and then after incubation of the test media for 24 hours at 37°C. This procedure entailed diluting a 1 ml sample of each medium with 1 ml of TISAB (ORION) which stabilizes the pH and ionic strength of the sample. All samples were compared to NaF standards (ORION).

Purity Checks of Organisms

To insure purity, the cultures at the beginning and end of each experiment were plated on blood and Mitis salivarius agar (Difco) and the plates were visually inspected for contaminants.

MIC/MLC Determination of Test Agents

To rule out the possibility that various fluorides or controls may have antiplaque properties at low concentrations due to their ability to kill or completely inhibit growth of plaque forming bacteria; the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of these agents (Barry, 1976) was determined.

S. mutans NCTC 10449S was adapted for growth in TS basal medium and 0.1 ml of the adapted strain was transferred to 10 ml of the medium. The turbidity of the culture was standardized and diluted 1:200 to produce an inoculum containing approximately $5.0X10^5$ CFU/ml. A 1.0 ml sample of the potential antimicrobial agents, SnF_2, $SnCl_2$, TiF_4, Na_2SnF_6 and NaF was serially twofold diluted in 1 ml of uninoculated TSB (for example, see

				10 ml		10 ml		10 ml
Refrig. Stock Culture S. mutans NCTC 10449S	24 h. ---→	BBL Thio	24 h. ---→	Jordan's 5% suc.	18 h. ---→	Jordan's 5% suc.	18 h. ---→	test groups

Figure 2. Procedure for adapting cultures of S. mutans to Jordan's medium supplemented with 5% sucrose. 0.1 ml of culture was transferred to uninoculated tubes each time.

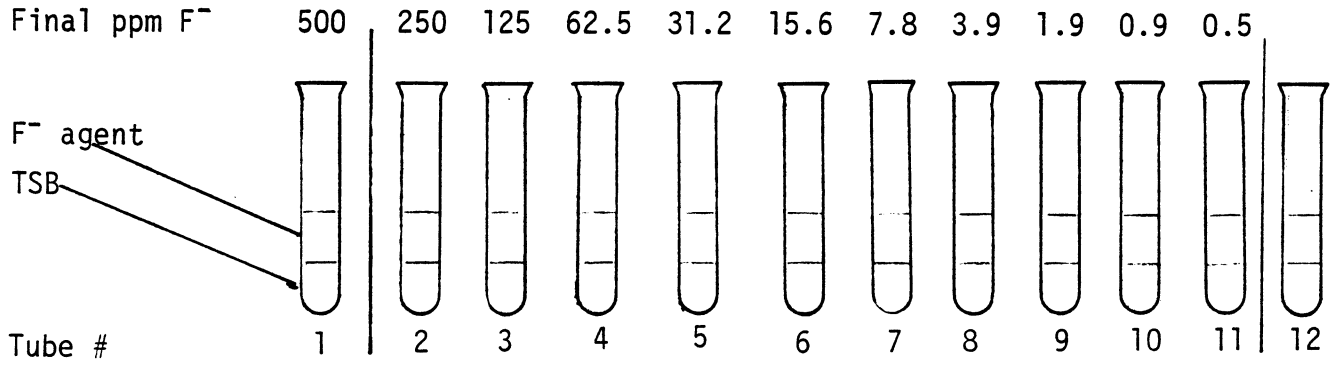

Figure 3. Example of a MIC and MLC dilution. First, 1.0 ml TSB is added to all tubes. Then, test agents are added to tube #1 and subsequently diluted with TSB starting with tube #2 and ending at tube #11. Tubes #1 and #12 are the uninoculated and inoculated controls. After serial dilution of the test agents, a 1.0 ml inoculum of standardized suspension is added to all tubes except #1.

Figure 3). To these tubes containing the serial dilutions, 1 ml of the standardized cell suspension was added. The tubes containing the various dilutions of antimicrobial agent plus the inoculum were mixed by vortexing, incubated at 37°C for 16-18 hours and read using a Spectronic 20 spectrophotometer (Bausche and Lomb, Rochester, NY).

Because of the potential precipitation of some test agents, the visual appearance of positive and negative control tubes was also considered before deteriming the MIC end points. Negative bacterial growth (MIC) was defined as the absence of turbidity or clumped deposits attached to the walls of the culture vial. The MLC was determined by transferring 0.1 ml of broth from each tube of broth showing no growth to blood and mitis salivarius agar plates. The plates were inspected for growth of S. mutans after 2 days incubation at 37°C.

Alteration in Bacterial Growth Due to Low Levels of Fluoride

To determine if the fluoride compounds or controls added to Jordan's medium affected the growth of S. mutans NCTC 10449S, growth curves were established and compared in the presence of NaF, SnF_2, Na_2SnF_6, TiF_4 at 10 or 5 ppm F added to this media. Additionally, $SnCl_2$ equimolar with respect to the tin in 10 or 5 ppm F as SnF_2 was used to observe any effect of the cation on growth. For this procedure, duplicate tubes containing the appropriate compound were inoculated with 0.1 ml of the organism, and growth rate was determined at 1 hour intervals using the spectrophotometer at 600 nm. Cultures were maintained at 37°C in a Thermomix 1420 Shaker Water Bath (B. Brawn, West Germany) oscilating at 60 cpm and a transit of 2.5 cm. The experiment was terminated at 24 hours because of the constancy of O.D. readings recorded after this time.

Also, initial and terminal pH readings were taken on all cultures. Controls for each agent tested were uninoculated Jordan's medium containing the corresponding agent at the same concentration. The fluoride test agents, $SnCl_2$ and H_2O (control) media were compared for their effect on doubling time and growth yield of S. mutans.

Alteration in Bacterial Acid Production Due to Low Levels of Fluoride or Controls

Into tubes containing 25 ml of Jordan's medium supplemented with 5% sucrose, NaF, SnF_2, Na_2SnF_6 and TiF_4 was added to produce 10 or 5 ppm F. $SnCl_2$ (Sn equal to Sn in SnF_2 at 10 or 5 ppm F) was also tested to observe the effect of the tin ion alone on bacterial acid production. An appropriate volume of deionized water was added to another tube containing growth medium to serve as the control. The tubes were inoculated with the adapted S. mutans strain. Culture tubes were then incubated in a shaker bath (37 C, 60 cpm). Samples were removed from the tubes for pH measurements at intervals during a 48 hour period. The mean pH of duplicate samples for each test agent was plotted against time. Samples were compared for differences in pH drop and terminal pH.

DNA/Glucan Analysis

Jordan's medium supplemented as previously described with 10 ppm F as NaF, SnF_2, Na_2SnF_6, TiF_4 or 10 ppm Cl as $SnCl_2$ (Sn = Sn in 10 ppm F SnF_2) was placed into tubes containing the suspended enamel cylinders and inoculated with 0.1 ml of an adapted S. mutans culture. Sterile deionized water served as the control agent. The enamel specimens were transferred after 24 hours to fresh Jordan's medium supplemented with 5% sucrose and containing the appropriate test agent or control (Figure 4).

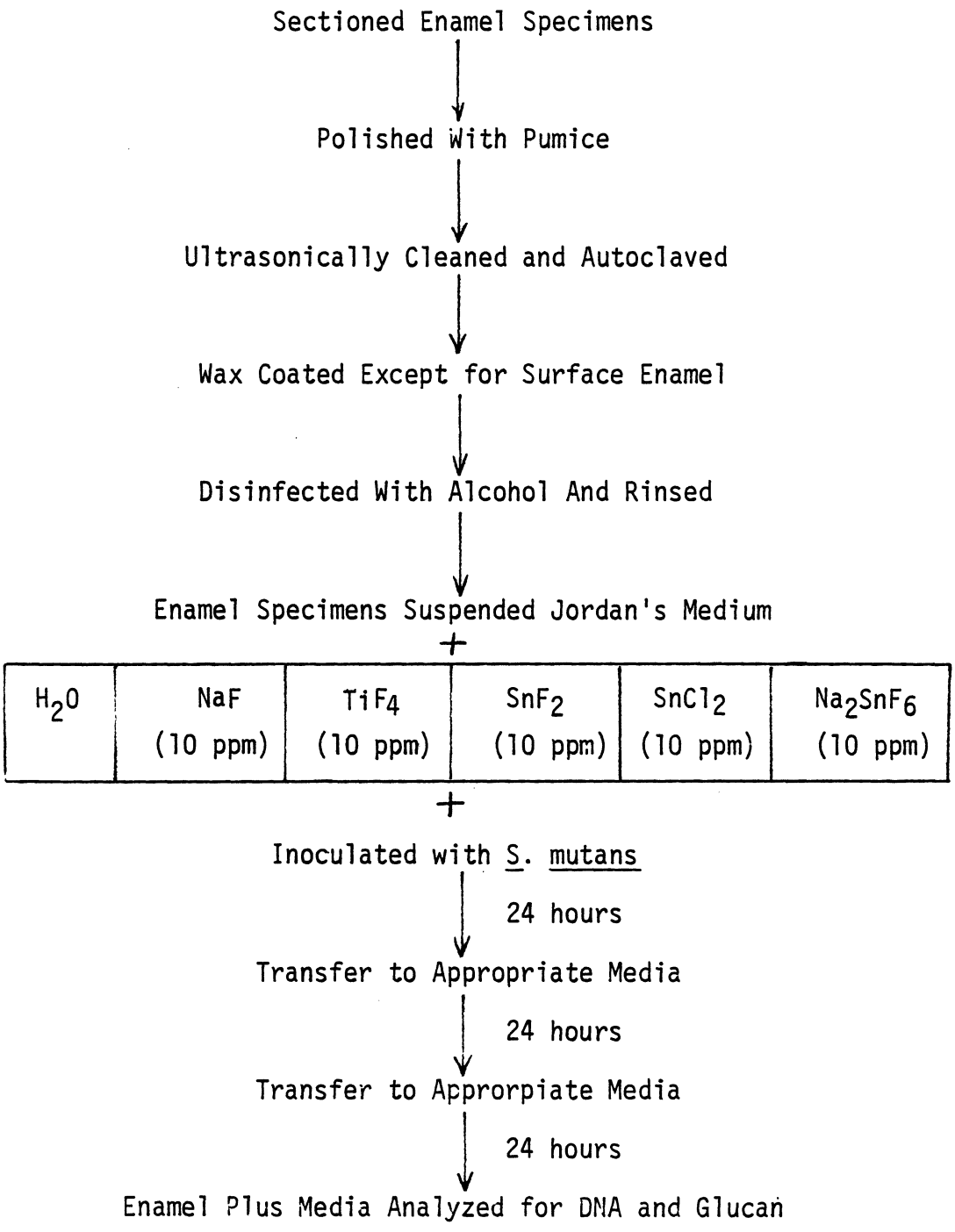

FIGURE 4. Experimental design for DNA and Glucan Analysis.

After 3 days growth in the appropriate media, the wax was removed from each enamel slab leaving only the bacteria attached to the surface enamel. The enamel specimens were then sonified using a Bronson Model w 185 sonifier (Heat Systems Ultrasonics, Plainview, NY) with a microprobe tip in deionized water for 30 seconds at 50 watts with the output at 4.

After washing and centrifugation (9000 x G, 10 minutes 0°C) of the bacterial sample three times, the sample was resuspended in a known volume of deionized water. A sample of the suspended cells and the spent supernatant fluid was saved for glucan analysis (Figure 5). The remainder of the suspended pellet was added to 0.5 N perchloric acid (70°C, 30 minutes) to extract the bacterial DNA for quantitation of bacterial mass (Ogur and Rosen, 1950). Also, the medium from which the enamel specimens were removed after the final transfer was prepared to analyzed DNA and glucan content.

After neutralization with KOH, DNA was analyzed (Burton, 1956) using a 2-deoxy-D-ribose as standard. DNA analysis involved centrifuging the sample as described. One ml of the hot PCA extract was combined with 2 ml of diphenylamine reagent, mixed by vortexing, covered, and incubated at 37 C overnight. 2-deoxy-ribose standards and blanks were also treated in like fashion. Optical densities of the samples and standards were read at 600 nm with a Model 300 N spectrophotometer (Gilford Instrument Laboratories, Columbia, MD) and converted to μg DNA/ml culture medium. Samples and standards were analyzed in duplicate.

All samples were prepared to quantitate glucan (for procedure, see Figure 5). Glucose was determined using the glucose oxidase reaction on neutralized (pH 7) hot acid hydrosylates (4N H_2SO_4, 2h, 100°C) of S. mutans glucan. Two ml of glucostat reagent (Worthington Biochemical, Freehold,

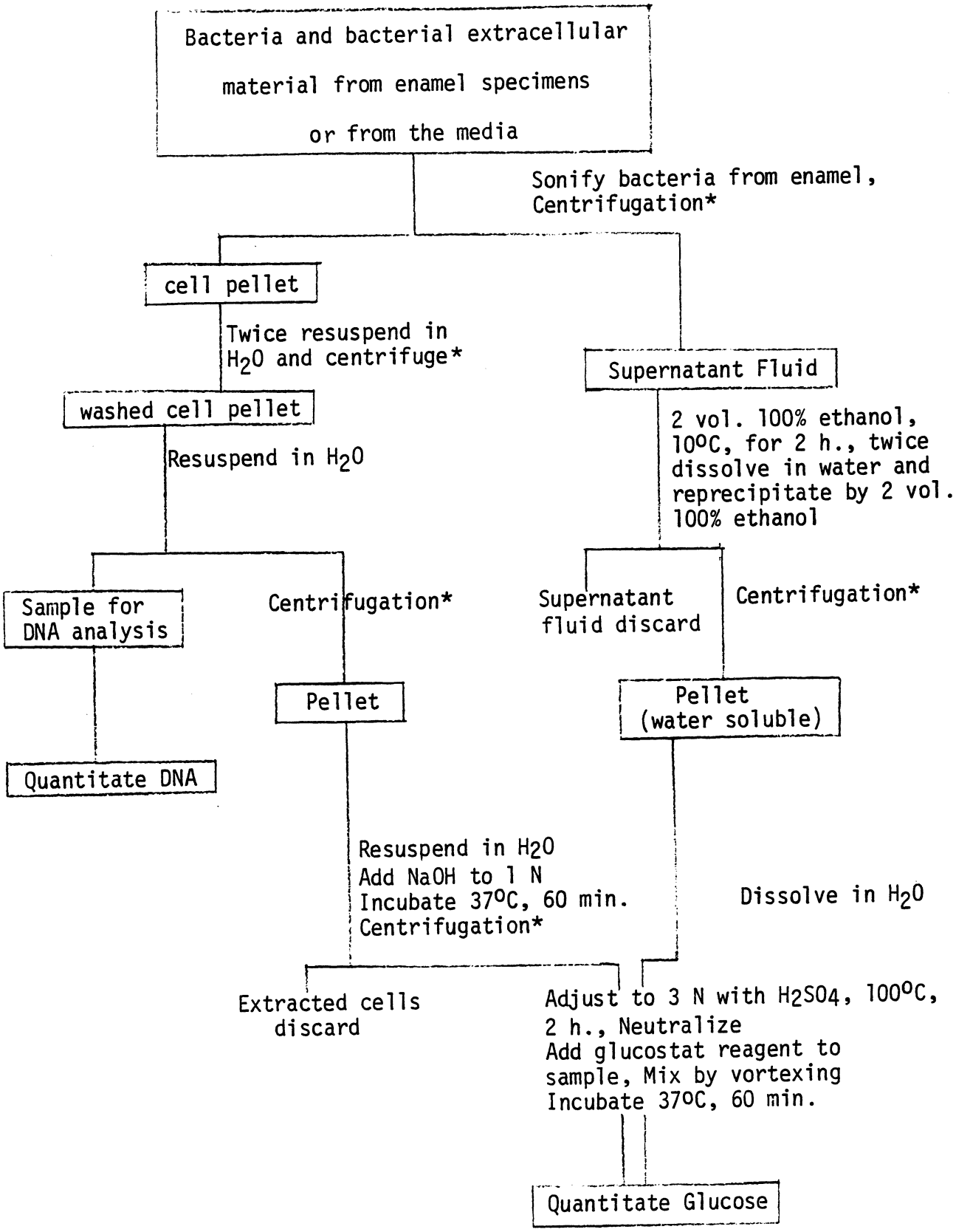

Figure 5: Flow diagram of the preparation of cells and culture liquor for glucose and DNA quantitations.

*All centrifugations: 9000 x g, 10 min. 0°C

NJ) dissolved in 500 mM tris phosphate buffer (pH 7) was added to 1 ml of prepared samples and read at 410 nm after incubation for 60 minutes at 37°C. Hydrolyzed commercial dextran ranging from 0 to 50 µg/ml served as standards (Freedman and Tanzer, 1974; Freedman and Coykendall, 1975). All samples and standards were analyzed in duplicate.

Determination of Enamel Surface Area

The surface area of the enamel slabs exposed to the various test agents or controls was calculated by placing black and white photographic negatives, determined to be a 1:1 representation of the enamel specimens, over mm^2 blocked graph paper. The number of mm^2 blocks contained within the outline of the enamel specimen negative is equivalent to the surface area of the specimen. The surface area of the enamel exposed to each test agent was used in determining µg DNA/mm^2 enamel and µg glucan/mm^2 enamel ratios.

Atomic Absorption Spectrophotometry

After 3 days growth, the bacteria on wires of each treatment group (Figure 1) were pooled into a pre-weighed centrifuge tube, pelleted by centrifugation, and excess water removed. Samples were dried for 3 days at 70°C and the tubes re-weighed. After the dry weights of the harvested cells were calculated, the samples were suspended in known quantities of 10% HCl. Tin standards ($SnCl_2$, Alfa Chemical, Danvers, MA) were prepared at 0.1, 0.5, 1.0, 10.0, 25.0, 35.0, and 50.0 ppm by dilution with 10% HCl. Tin in the plaque samples and in the standards were measured in triplicate and compared using a Model 403 atomic absorption spectrophotometer (Perkin-Elmer, Stamford, CT) equipped with a AGA-74 graphite furnace. A deuterium discharge lamp was used to correct for non-atomic absorption signals.

Statistical Analysis

For statistical verification of DNA and glucan test results, the mean and standard deviation of the second DNA/glucan experiment (3 samples, test or control group) were calculated. Analysis of variance indicated statistically significant differences ($p \leq .01$). Individual comparisons of the test agents on controls was performed using the Scheffe procedure (Scheffe, 1953) to establish homogeneous subsets at the .01 level.

Electron Microscopic Evaluation

To observe structural changes in bacterial attachment due to the fluoride or tin compounds, .030" stainless steel wires suspended in the various fluoride supplemented Jordan's media, were inoculated with S. mutans, and incubated for 3 days, then processed for electron microscopic observation. Wires incubated in inoculated Jordan's medium which did not have the addition of the test compounds were the control. After the three day incubation period, the wires and attached microorganisms were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4, 390 mOsm). The fixed microorganisms were mechanically dislodged from the stainless steel wires, postfixed in 1% osmium tetroxide in veronal buffer (pH 7.3) (Warshawsky and Moore, 1967), and washed in phosphate buffer. The specimens were dehydrated in acetone and embedded in Spurr's epoxy resin (Spurr, 1969).

After polymerization of the specimen at 70°C for 24 hours, thin sections of each specimen for electron microscopic observation were cut with a Sorvall MT2B ultramicrotome (Sorvall Company, Norwalk, CT) using a Dupont diamond knife (Dupont Company, Delaware, MD). Silver-gold colored sections were either examined stained with aqueous uranyl acetate

followed by lead citrate (Venable and Coggeshall, 1965) or examined unstained at 90kV with a Zeiss EM 10 electron microscope.

At least 3 areas from each specimen were observed to minimize subjective misinterpretation. Comparison between samples were made using 6 criteria: 1) presence of extracellular material; 2) affinity and attachment of bacteria to each other; 3) structural changes of the bacterial cell wall; 4) internal changes of cells; 5) presence of electron dense granules on or in the cells; and 6) presence of polyphosphate (electronlucent holes) within the bacteria.

Furthermore, a representative electronmicrograph from each sample at a standard magnification (5,000 x) was used to semi-quantitate certain observations. This procedure entailed counting the number of intracellular and extracellular electron dense granules and the number of electron lucent holes on the selected micrograph from each specimen. The ratio of these structures to the bacteria present in the micrograph was then determined by dividing these observations by the total number of bacteria found on each micrograph.

Results

Determination of Fluoride Levels in Growth Medium

Samples of the media supplemented with the various fluoride compounds at 10 ppm F (W/V) were analyzed for fluoride ion activity immediately after the additions of the fluoride compounds, after incubation of uninoculated media for one day, and after one day's growth of S. mutans cultures in the media.

The water and $SnCl_2$ supplemented media demonstrated essentially no fluoride ion present. NaF, Na_2SnF_6 and SnF_2 supplemented media has 10 ppm F present in fresh medium. After 24 hours incubation, however, all three of these agents showed a decrease of approximately 1 ppm F in both inoculated and uninoculated medium. TiF_4 was the only compound tested that did not show fluoride levels equal to theoretical levels. A theoretical level of 10 ppm F gave an actual reading of only 2.3 ppm F in fresh medium and after 24 hours incubation of inoculated and uninoculated medium (Table 1).

MIC/MLC Determinations

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) tests showed that SnF_2 had the lowest MIC and MLC, 60 ppm F and 125 ppm F respectively (Tables 2 and 7). TiF_4 had a MIC of 550 $\pm$ 25 ppm F and a MLC of 575 + 25 ppm F (Tables 3 and 7); NaF had a MIC of 300 ppm F and a MLC of 3000 ppm F (Tables 4 and 7); Na_2SnF_6 had a MIC of 600 ppm F and MLC of 675 ppm F (Tables 5 and 7). $SnCl_2$ was found to have a MIC of 200 ppm Cl and a MLC of 225 ppm Cl (Tables 6 and 7).

With respect to the tin ion concentration of the compounds tested, SnF_2 had a MIC of 180 ppm Sn while $SnCl_2$ and Na_2SnF_6 both had a MIC of

	ppm F		
	Fresh uninoculated medium	Uninoculated medium after incubation for 24 hr at 37° C	Inoculated medium after 24 hr growth of S. mutans NCTC 10449 S at 37°C
Control	< 0.3	< 0.3	< 0.3
$SnCl_2$	< 0.3	< 0.3	< 0.3
TiF_4	2.3	2.3	2.3
NaF	10.0	9.0	9.0
Na_2SnF_6	10.0	8.8	8.8
SnF_2	10.0	9.0	9.0

Table 1. Fluoride ion concentration (ppm) in inoculated and uninoculated Jordan's medium supplemented with fluoride compounds, $SnCl_2$ or water control.

ppm F	Culture Vial (MIC)	MSS plate (MLC)	BA plate (MLC)
250	NG, P	NG	NG
125	NG, P	NG	NG
100	NG, P	<<G	<<G
90	NG, P	<G	<G
80	NG, P	<G	<G
70	NG, P	<G	<G
60	NG, P	G	G
50	G, P	G	G
40	G, P	G	G
30	G, P	G	G
20	G, P	G	G
10	G, P	G	G
5	G, P	G	G
2.5	G, NP	G	G
1.8	G, NP	G	G
0.9	G, NP	G	G
0.5	G, NP	G	G

Key: NG = No Growth
<G = Less Growth
G = Growth
P = Precipitate
NP = No Precipitate

Table 2: Dilutions of SnF_2 ranging from 0.5 to 250 ppm F were evaluated for a minimum inhibitory concentration (MIC) and a minimum lethal concentration (MLC) against S. mutans NCTC 10449S. A MIC for SnF_2 of 60 ppm F was determined from the culture vial of the lowest dilution showing no growth; a MLC for SnF_2 of 125 ppm F was determined by the lowest concentration fluoride containing vial on BA and MSS plates showing no growth. None of the uninoculated control vials or plates exhibited growth. A milky white precipitate was present in both the uninoculated control and test dilutions at $\leq$ 5 ppm F.

ppm F	Culture Vial (MIC)	MSS plate (MLC)	BA plate (MLC)
1000	NG, P	NG	NG
900	NG, P	NG	NG
800	NG, P	NG	NG
700	NG, P	NG	NG
650	NG, P	NG	NG
625	NG, P	NG	NG
575±25	NG, P	NG	NG
550±25	NG, P	G	G
500	G, P	G	G
475	G, P	G	G
450	G, P	G	G
400	G, P	G	G
300	G, P	G	G
200	G, P	G	G
100	G, P	G	G

Key: NG = No Growth
G = Growth
P = Precipitate

Table 3: Dilutions of TiF_4 ranging from 100 to 1000 ppm F were evaluated for MIC and MLC against S. mutans. In all test runs, the MIC and MLC was found within the 525 to 600 ppm F range. The MIC of TiF_4 was 550 ± 25 ppm F; the MLC of TiF_4 was 575 ± 25 ppm F. None of the uninoculated control vials or plates exhibited growth and no contamination was noted on culture plates of test dilutions. A cloudy gray-white precipitate was present in all test and uninoculated control dilutions.

ppm F	Culture Vial (MIC)	MSS plate (MLC)	BA plate (MLC)
5000	NG	NG	NG
4500	NG	NG	NG
4000	NG	NG	NG
3500	NG	NG	NG
3250	NG	NG	NG
3000	NG	NG	NG
2750	NG	<<<G	<<<G
2500	NG	<<<G	<<<G
2000	NG	<<G	<<G
1500	NG	<<G	<<G
1000	NG	<G	<G
900	NG	<G	<G
800	NG	<G	<G
700	NG	<G	<G
600	NG	<G	<G
500	NG	<G	<G
400	NG	G	G
350	NG	G	G
300	NG	G	G
250	G	G	G
200	G	G	G
150	G	G	G
100	G	G	G
50	G	G	G

Key: NG = No Growth
<G = Less Growth
G = Growth

Table 4: Dilutions of NaF ranging from 50 to 5000 ppm F were evaluated for MIC and MLC against S. mutans. The MIC for NaF was 300 ppm F; the MLC for NaF was 3000 ppm F. Plated dilutions of NaF ranging from 500 to 2750 ppm F exhibited decreased growth on BA and MSS plates after 24 hours incubation at 35°C. None of the uninoculated control vials or plates exhibited growth. No contamination was noted on culture plates of test dilutions.

ppm F	Culture Vial (MIC)	MSS plate (MLC)	BA plate (MLC)
1000	NG	NG	NG
900	NG	NG	NG
800	NG	NG	NG
700	NG	NG	NG
675	NG	NG	NG
650	NG	<G	<G
625	NG	<G	<G
600	NG	G	G
575	<G	G	G
550	G	G	G
525	G	G	G
500	G	G	G
475	G	G	G
400	G	G	G
300	G	G	G
200	G	G	G
100	G	G	G

Key: NG = No Growth
<G = Less Growth
G = Growth

Table 5: Dilutions of Na_2SnF_6 ranging from 100 to 1000 ppm F were evaluated for MIC and MLC against S. mutans. The MIC of Na_2SnF_6, was 600 ppm F; the MLC of Na_2SnF_6, was 675 ppm F. None of the uninoculated control vials or plates exhibited growth. No contamination was noted on culture plates of test dilutions.

ppm Sn*	Culture Vial (MIC)	MSS plate (MLC)	BA plate (MLC)
1000	NG, P	NG	NG
900	NG, P	NG	NG
800	NG, P	NG	NG
700	NG, P	NG	NG
600	NG, P	NG	NG
500	NG, P	NG	NG
400	NG, P	NG	NG
300	NG, P	NG	NG
250	NG, P	NG	NG
200	NG, P	G	G
175	<G, P	G	G
150	G, P	G	G
125	G, P	G	G
100	G, P	G	G
75	G, P	G	G
50	G, P	G	G

Key: NG = No Growth
<G = Less Growth
G = Growth
P = Precipitate

*ppm equivalent to Sn in a SnF_2 solution of equal concentration

Table 6: Dilutions of $SnCl_2$ ranging from 50 to 1000 ppm Cl, were evaluated for MIC and MLC. The MIC of $SnCl_2$, was 200 ppm Cl; the MLC of $SnCl_2$, was 225 ppm Cl. None of the uninoculated control vials or plates exhibited growth. No contamination was noted on culture plates of test dilutions. A cloudy yellow-white precipitate was present in all uninoculated control and test dilution vials.

Test Compound	MIC		MLC	
	ppmF^-	ppmSn	ppmF^-	ppmSn
SnF_2	60	180	125	375
$SnCl_2$	(200 ppmCl^-)	600	(225 ppmCl^-)	675
Na_2SnF_6	600	600	675	675
NaF	300		3000	
TiF_4	550 $\pm$ 25		575 $\pm$ 25	

Table 7: Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of various fluoride compounds, $SnCl_2$ or H_2O (control) on S. mutans NCTC 10449S.

600 ppm Sn, and SnF_2 had a MLC of 375 Sn while $SnCl_2$ and Na_2SnF_6 had a MLC of 675 ppm Sn (Table 7).

Alterations in Bacterial Acid Production Due to Low Levels of Fluoride

Acid production of S. mutans incubated in medium supplemented with various fluoride compounds at 10 and 5 ppm F, i.e., at concentrations well below the levels determined to inhibit growth (MIC), was measured and compared with zero fluoride controls (Figures 6 and 7).

SnF_2, NaF, and Na_2SnF_6 appear to be equally effective in altering the terminal pH of the S. mutans cultures both at 5 ppm F and 10 ppm F concentrations. At 5 ppm F of these compounds, the terminal pH of SnF_2, NaF and Na_2SnF_6 supplemented media was approximately pH 4.8 whereas the $SnCl_2$ supplemented media and water control had a terminal pH of 4.3. When the medium was supplemented with 10 ppm SnF_2, NaF, or Na_2SnF_6 the terminal pH only reached 5.0.

TiF4 at both 5 and 10 ppm F showed little or no effect on acid production. This may be because the actual levels of F in solution for this compound were found to be much lower than the theoretical levels.

All experiments were performed in duplicate runs and slight variations were averaged. Note that the addition of the test compounds did not produce an initial pH change of the media when compared to the control medium.

Alteration in Bacterial Growth Due to Low Levels of Fluoride

Alterations in growth rate of S. mutans in medium supplemented with various fluoride compounds at 10 and 5 ppm F or with H_2O or $SnCl_2$ were evaluated in order to observe the effect of these agents at concentrations well below the levels determined to inhibit growth (MIC) (Figures

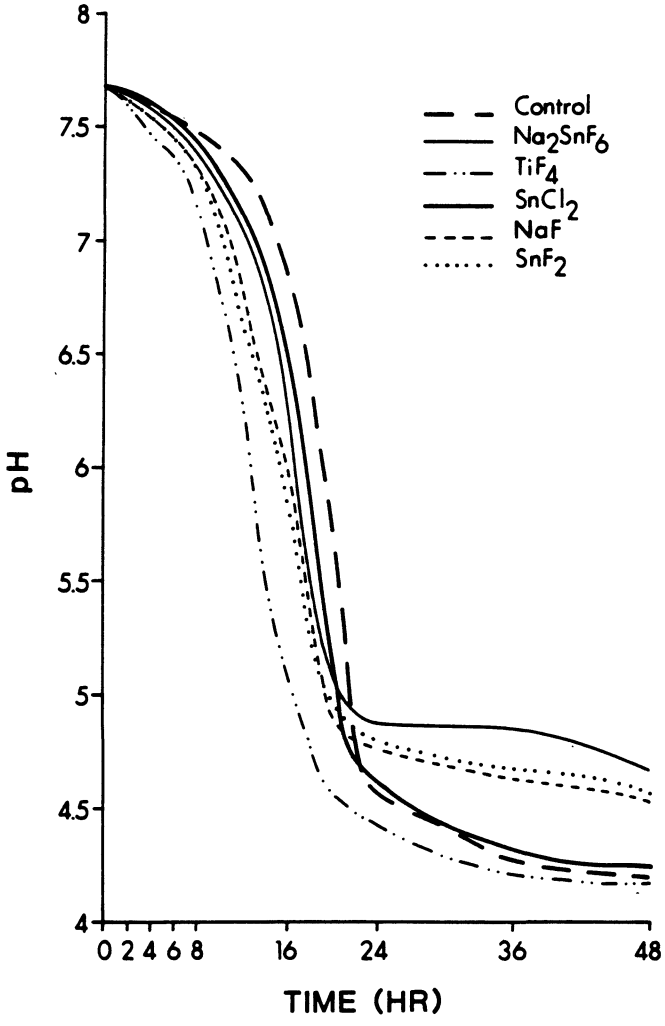

Figure 6: Acid production by S. mutans NCTC 10449S in medium supplemented with various fluoride compounds (5 ppm F). Control represents water added instead of a fluoride compound.

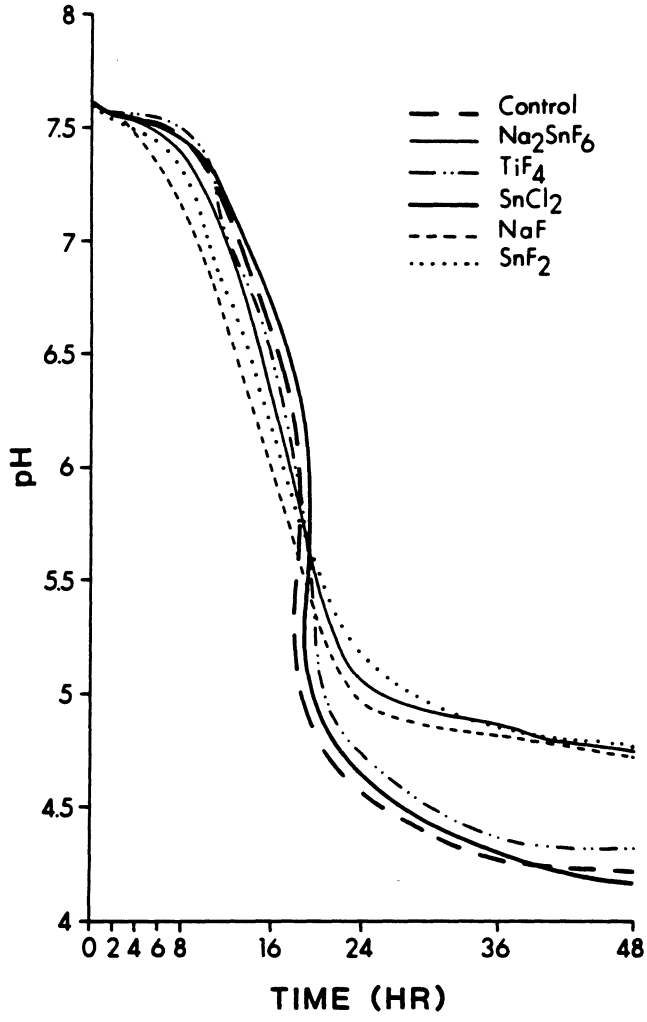

Figure 7: Acid production by S. mutans NCTC 10449S in medium supplemented with various fluoride compounds (10 ppm F). Control represents water added instead of a fluoride compound.

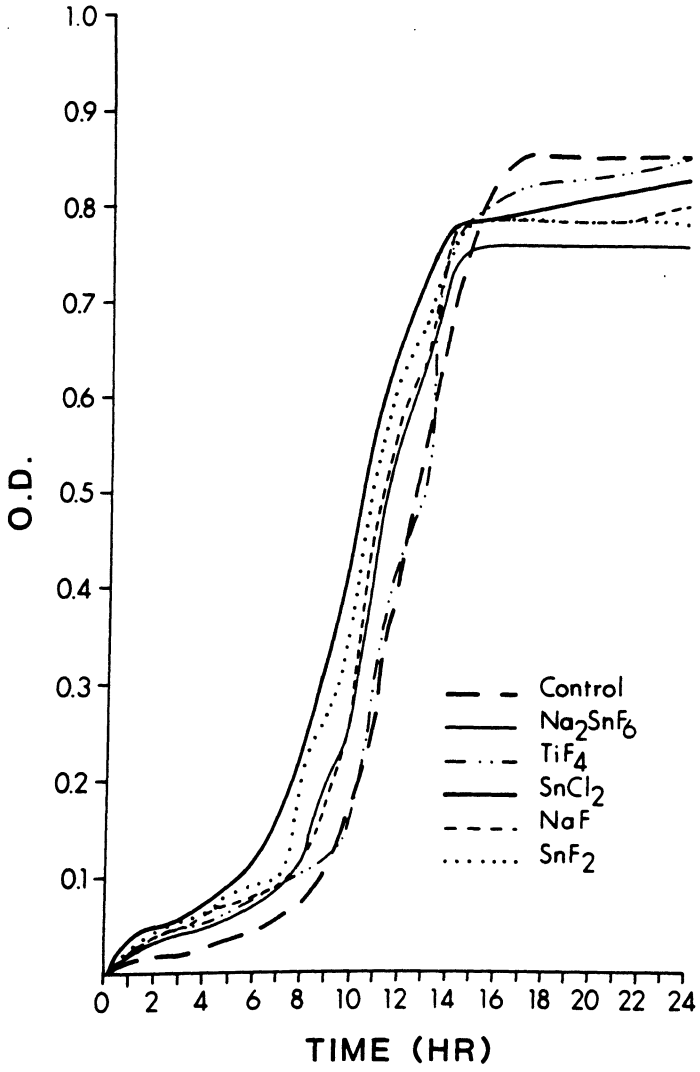

Figure 8: Growth of S. mutans NCTC 10449S in medium supplemented with various fluoride compounds (5 ppm F). Control represents water added instead of a fluoride compound.

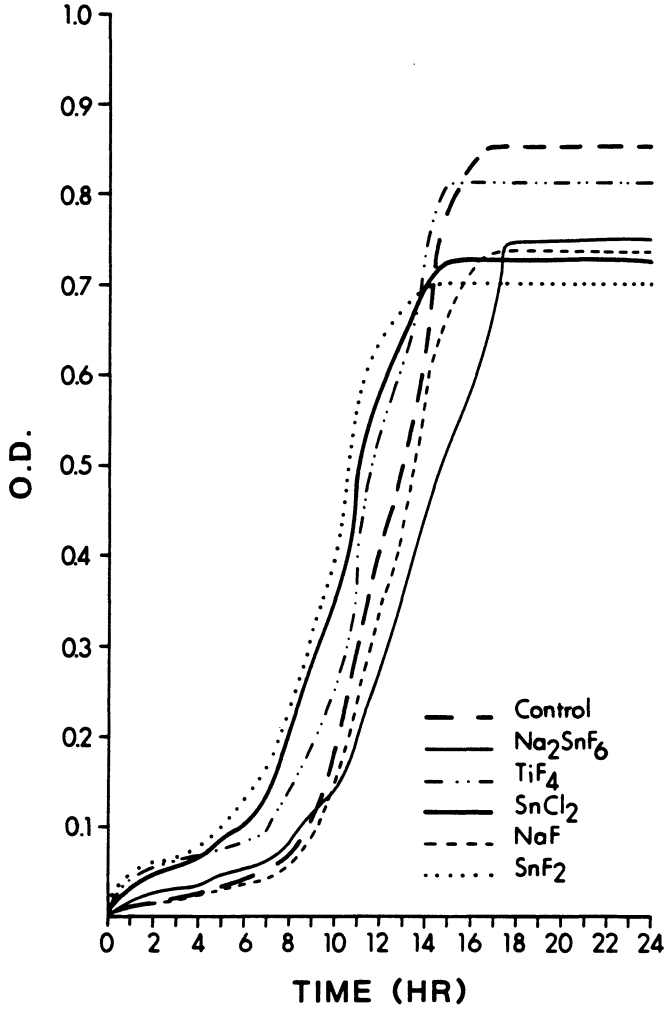

Figure 9: Growth of S. mutans NCTC 10449S in medium supplemented with various fluoride compounds (10 ppm F). Control represents water added instead of a fluoride compound.

8 and 9).

Growth curves, performed in duplicate, suggest little difference between the doubling times of the organisms exposed to the test agents at 5 or 10 ppm. At 5 ppm, no distinct difference in growth yields were seen between the control and the test compounds (Figure 8). However, at 10 ppm, the growth yield appears less in all media supplemented with either fluoride or tin compounds (Figure 9), with SnF_2 showing the lowest growth yield.

DNA/Glucan Analysis

DNA and glucan analyses were performed on the bacteria attached to suspended enamel specimens and on the bacteria present in the media from the third day of growth. This series of experiments was performed twice. The first experiment was done with samples run in duplicate (Tables 8-11) but in order to verify these results and to allow statistical appraisal using subset grouping through analysis of variance, it was necessary to repeat the experiment using three samples per test group (Tables 12-15).

Results of both experiments showed less DNA and alkali soluble glucan (ASG) attached to enamel in the presence of SnF_2, Na_2SnF_6, and NaF compared to other compounds tested, with SnF_2 showing the least (Table 8, 12). However, there was no significant difference in the μg ASG/μg DNA among these samples. This suggests that the lower ASG found in the fluoride test groups was due to the presence of fewer bacteria in these groups (Table 12).

When the total amount of DNA on the third day (attached bacteria from three day's growth and unattached bacteria from the third day's growth only) of the various test groups was compared for all medium containing fluoride test agents, there was less DNA/ml medium in the

presence of F then in its absence. The SnF_2 supplemented medium resulted in the least bacterial quantity (Table 9, 13). When total µg ASG/µg DNA ratios were evaluated, SnF_2, Na_2SnF_6 and NaF exhibited an increase in alkali soluble glucan compared to other test groups. The water soluble glucan (µg WSG/µg DNA) also showed the greatest increase in those cultures supplemented with SnF_2, NaF or Na_2SnF_6 (Tables 9, 13). The ASG present in the culture tube further demonstrated that increased ASG production of the unattached bacteria by S. mutans under the influence of SnF_2, NaF, or Na_2SnF_6, occurs (Tables 10, 14).

Tin Analysis

After 3 days' growth, the bacteria on the wires of each treatment group (Figure 1) were analyzed for tin using atomic absorption spectrophotometry. As expected, no tin was detected in the control, TiF_4, and NaF treatment groups. The bacteria grown in the presence of SnF_2, $SnCl_2$ and Na_2SnF_6 were found to contain tin. The plaque incubated in SnF_2 supplemented media had more tin per mg. dry plaque than those specimens cultured in $SnCl_2$ or Na_2SnF_6 supplemented medium (Tables 11, 15).

Electron Microscopy

Increases in extracellular material were observed in those S. mutans specimens cultured in the presence of fluoride as compared to the $SnCl_2$ or water control (Figures 10, 11, 13-15). This increased density of extracellular material was not, however, present in the TiF_4 group, presumably due to the low levels of fluoride in the growth media with this agent (Figure 14). The electron micrographs of those bacteria inoculated with SnF_2 also revealed distorted bacterial shapes and atypical coccal morphology as compared to the other groups (Figure 15).

Semiquantitative assessment to determine the percentage of cells having electron lucent holes (bacterial polyphosphate) and electron dense granules (tin) was performed on all specimens at a standard magnification (x 11,000). Electron lucent holes were found in 12% of those bacteria in the water control group, 12% in the Na_2SnF_6 group, 13% in the TiF_4 group and 19% in the NaF group. However, electron lucent holes were found in 36% of those bacteria in the $SnCl_2$ group and in 86% of those bacteria in the SnF_2 group. Quantitation of the electron dense granules in the $SnCl_2$ group and SnF_2 group revealed that 5% of the bacteria in the $SnCl_2$ test group had electron dense granules associated with the bacterial cell wall and 6% of the cells had electron dense granules within the cells. SnF_2 treated bacteria had granules on 1% of the cell walls and 19% within the cell.

μg DNA/mm^2 enamel*		μg ASG/mm^2 enamel		μg ASG/μg DNA	
Control	0.18 ± .03	Control	1.02 ± .01	NaF	7.06 ± .64
$SnCl_2$	0.18 ± .003	TiF_4	0.99 ± .05	TiF_4	6.31 ± .06
TiF_4	0.16 ± .02	$SnCl_2$	0.97 ± .12	SnF_2	5.79 ± .15
Na_2SnF_6	0.14 ± .02	NaF	0.86 ± .19	Na_2SnF_6	5.77 ± .27
NaF	0.12 ± .02	Na_2SnF_6	0.84 ± .07	Control	5.67 ± .30
SnF_2	0.09 ± .01	SnF_2	0.55 ± .06	$SnCl_2$	5.19 ± .73

Table 8: Experiment 1. Amount of bacteria (DNA) and alkali soluble glucan (ASG) adherent to enamel after three days' incubation of S. mutans NCTC 10449S in medium supplemented with various fluoride compounds (10 ppm F), $SnCl_2$, or H_2O (control).

Total µg DNA/ml*	
$SnCl_2$	4.6 ± 0.5
Control	3.7 ± 0.7
NaF	3.2 ± 0.1
Na_2SnF_6	3.1 ± 0.6
TiF_4	3.0 ± 0.6
SnF_2	2.3 ± 0.3

Total µg ASG/ml	
NaF	47.0 ± 6.8
Na_2SnF_6	44.2 ± 0.9
$SnCl_2$	35.8 ± 1.0
TiF_4	31.7 ± 3.5
SnF_2	31.4 ± 1.6
Control	30.7 ± 3.3

Total µg ASG/µg DNA	
NaF	14.8 ± 1.7
Na_2SnF_6	14.4 ± 2.4
SnF_2	13.3 ± 0.7
TiF_4	10.9 ± 0.9
Control	8.3 ± 0.6
$SnCl_2$	7.8 ± 0.04

Total µg WSG/ml	
SnF_2	28.9 ± 1.9
Na_2SnF_6	22.4 ± 0.6
NaF	20.9 ± 1.3
TiF	5.3 ± 1.1
$SnCl_2$	3.9 ± 2.3
Control	2.3 ± 1.2

Total µg WSG/µg DNA	
SnF_2	12.3 ± 2.0
Na_2SnF_6	7.3 ± 1.5
NaF	6.6 ± 0.2
TiF	1.8 ± 0.01
$SnCl_2$	0.9 ± 0.05
Control	0.6 ± 0.2

*Mean of 2 samples (DNA/ml culture media) ± S.D.

Table 9. Experiment 1. Total amount of bacteria (DNA), alkali soluble glucan (ASG), and water soluble glucan (WSG) which was adherent to enamel and present in the culture tube on the third day's incubation of S. mutans NCTC 10449S. Growth medium was supplemented with either fluoride compounds (10 ppm F), $SnCl_2$ or H_2O (control).

	µg ASG/ml*
Na_2SnF_6	30.5 ± 2.1
NaF	28.6 ± 2.7
SnF_2	21.4 ± 0.4
TiF_4	14.7 ± 0.4
$SnCl_2$	14.1 ± 3.3
Control	11.7 ± 0.5

*Mean of 2 samples (µg ASG/ml culture media) ± S.D.

Table 10. Experiment 1. Alkali soluble glucan (ASG) present in the culture tube medium on the third day's incubation of S. mutans NCTC 10449S in medium supplemented with various fluoride compounds (10 ppm F), $SnCl_2$ or H_2O (control).

	Plaque Dry Weight* (mg)	Sn /Total Sample (ppm)	Sn /mg Plaque (μg)
Control	0.7	N.D.**	N.D.
Na_2SnF_6	0.7	19	13
TiF_4	1.3	N.D.	N.D.
$SnCl_2$	0.8	42	26
NaF	0.7	N.D.	N.D.
SnF_2	0.7	66	48

*Pooled plaque from 2 samples.
**None detected.

Table 11. Experiment 1. Bacteria harvested from the wires suspending the enamel specimens in each treatment group. Samples were pooled, dried, and analyzed for tin using Atomic Absorption Spectrophotometry. The limit of detection of total Sn using this method was <1 ppm.

	μg DNA / mm^2 Enamel*	Subsets**		μg ASG / mm^2 Enamel*	Subsets		μg ASG / μg DNA	Subsets
Control	0.17 ± .02		TiF_4	1.35 ± .43		TiF_4	9.02 ± 1.24	
$SnCl_2$	0.15 ± .00		$SnCl_2$	1.19 ± .05		$SnCl_2$	7.42 ± 0.85	
TiF_4	0.14 ± .03		Control	1.10 ± .16		NaF	7.18 ± 0.70	
Na_2SnF_6	0.12 ± .02		NaF	0.83 ± .19		SnF_2	6.72 ± 1.28	
NaF	0.11 ± .02		Na_2SnF_6	0.62 ± .04		Control	6.39 ± 0.59	
SnF_2	0.05 ± .00		SnF_2	0.32 ± .09		Na_2SnF_6	5.37 ± 0.95	

* Mean of 3 samples ± S.D.
** Homogeneous subsets using Analysis of Variance with Scheffe procedure ($p \leq .01$)

Table 12. Experiment 2. Amount of bacteria (DNA) and alkali soluble glucan (ASG) adherent to enamel after three days' incubation of S. mutans NCTC 10449S in medium supplemented with various fluoride compounds (10 ppm F), $SnCl_2$ or H_2O (control).

Total μg DNA/ml *		Subsets**
Control	3.56 ± .41	
NaF	2.87 ± .86	
Na_2SnF_6	2.72 ± .75	
TiF_4	2.66 ± .57	
$SnCl_2$	2.52 ± .20	
SnF_2	1.27 ± .11	

Total μg ASG/ml		Subsets
Na_2SnF_6	58.1 ± 12.0	
NaF	55.2 ± 2.2	
SnF_2	55.1 ± 13.0	
TiF_4	33.0 ± 7.9	
Control	32.9 ± 3.8	
$SnCl_2$	21.7 ± 2.6	

Total μg ASG/μg DNA		Subsets
SnF_2	33.2 ± 3.6	
Na_2SnF_6	23.3 ± 10.7	
NaF	20.2 ± 5.3	
TiF_4	12.4 ± 0.45	
Control	9.3 ± 1.3	
$SnCl_2$	8.6 ± 0.81	

Total μg WSG/ml		Subsets
NaF	57.5 ± 7.8	
SnF_2	56.6 ± 10.5	
Na_2SnF_6	47.2 ± 8.3	
TiF_4	17.7 ± 4.8	
Control	15.5 ± 0.9	
$SnCl_2$	10.9 ± 4.0	

Total μg WSG/μg DNA		Subsets
SnF_2	45.0 ± 11.3	
NaF	21.4 ± 7.2	
Na_2SnF_6	14.1 ± 3.9	
TiF_4	6.7 ± 1.6	
$SnCl_2$	4.2 ± 1.3	
Control	4.0 ± 0.48	

* Mean of 3 samples ± S.D.

** Homogeneous subsets using Analysis of Variance with Scheffe procedure ($p \leq .01$)

Table 13. Experiment 2. Total amount of bacteria (DNA), alkali soluble glucan (ASG), and water soluble glucan (WSG) which was adherent to enamel and present in the culture tube on the third day's incubation of S. mutans NCTC 10449S. Growth medium was supplemented with either fluoride compounds (10 ppm F), $SnCl_2$ or H_2O (control).

	μg ASG/ml*	Subsets**
Na_2SnF_6	47.9 ± 11.8	|
NaF	42.3 ± 1.3	|
SnF_2	35.8 ± 3.7	|
TiF_4	11.2 ± 3.6	|
Control	11.2 ± 5.0	|
$SnCl_2$	3.1 ± 0.8	|

* Mean of 3 samples ± S.D.

** Homogenous subsets using Analysis of Variance with Scheffe procedure ($p \leq .01$)

Table 14. Experiment 2. Alkali soluble glucan (ASG) present in the culture tube medium on the third day's incubation of S. mutans NCTC 10449S in medium supplemented with various fluoride compounds (10 ppm F), $SnCl_2$ or H_2O (control).

	Plaque Dry Weight (mg)	Sn°/ Total Sample (ppm)	Sn°/ mg. plaque (μg)
Control	1.8	N.D.	N.D.
Na_2SnF_6	1.5	6	4
TiF_4	3.0	N.D.	N.D.
$SnCl_2$	2.4	48	20
NaF	1.7	N.D.	N.D.
SnF_2	1.4	47	34

N.D. - None Detected

Pooled plaque from 3 samples.

Table 15. Experiment 2. Bacteria harvested from the wires suspending the enamel specimens in each treatment group. Samples were pooled, dried, and analyzed for tin using Atomic Absorption Spectrophotometry. The limit of detection of total Sn using this method was <1 ppm.

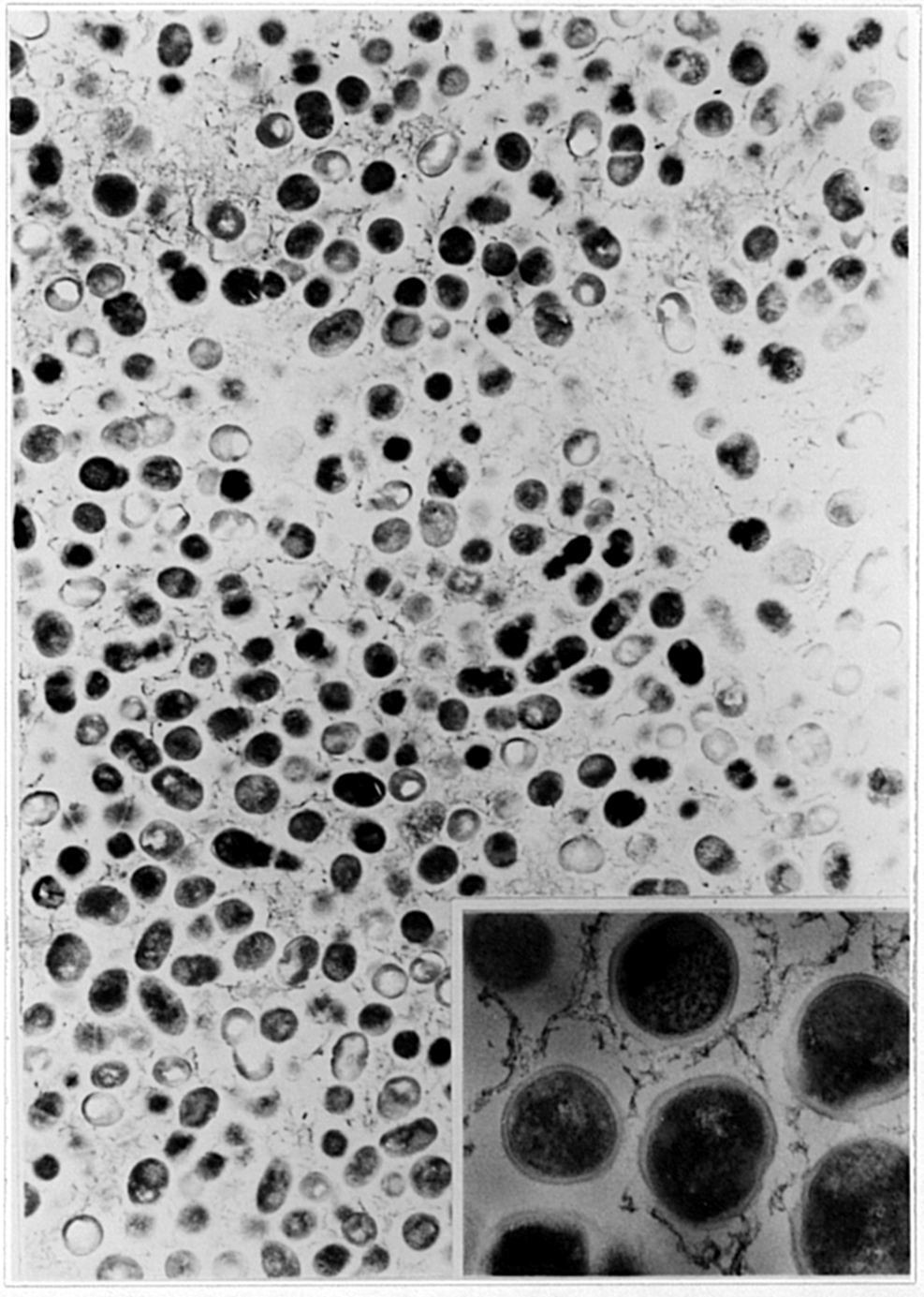

Figure 10: Transmission electron micrograph of S. mutans NCTC 10449S incubated for 3 days in Jordan's medium supplemented with 5% sucrose (H_2O control). Coccal bacteria in an extracellular matrix is evident at low magnification, x 11,000. High magnification demonstrates typical gram positive cocci with associated extracellular material, x 55,000.

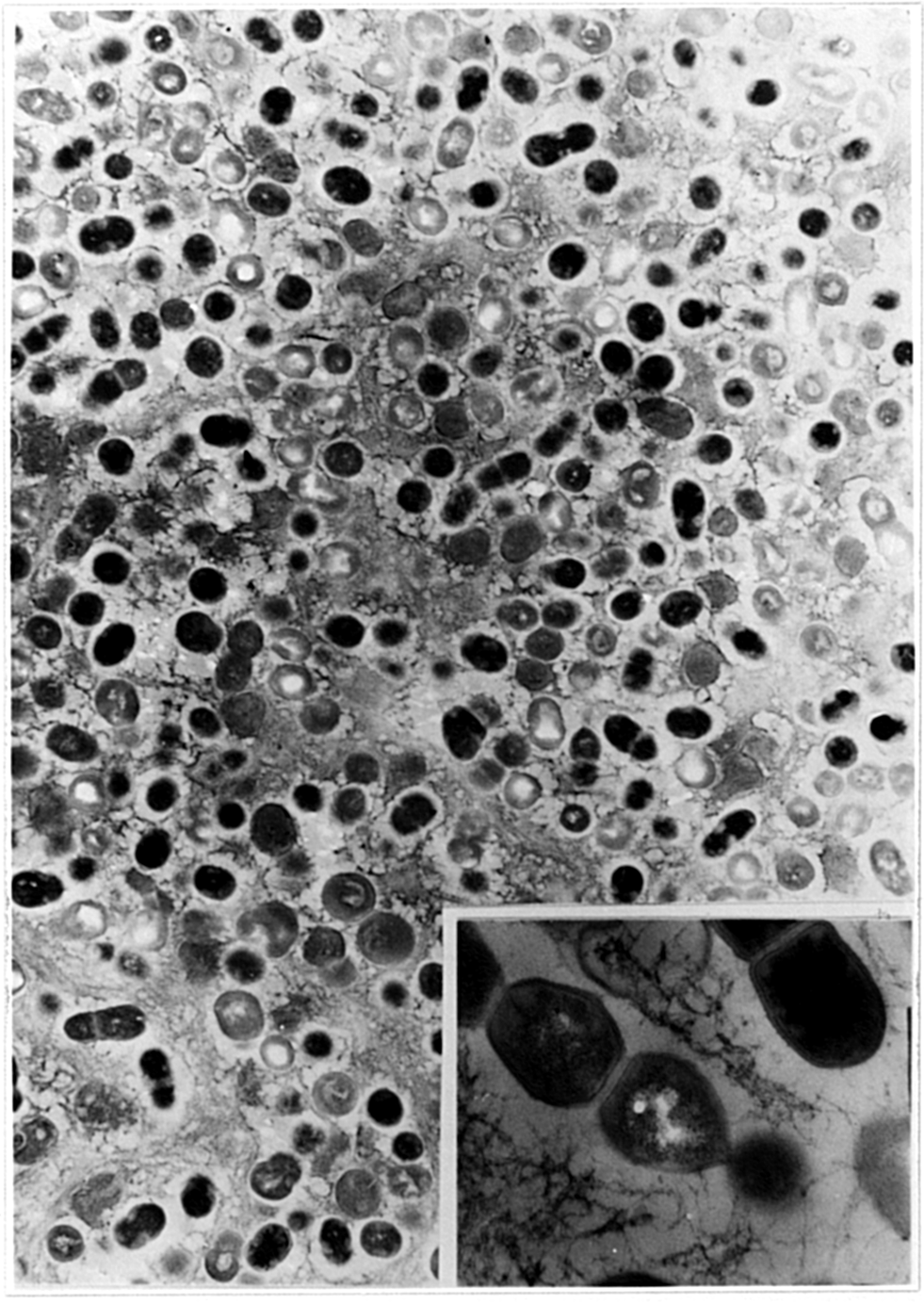

Figure 11: T.E.M. of S. mutans incubated for 3 days in Jordan's medium containing 5% sucrose and NaF (10 ppm F). More extracellular material is apparent than in the control photomicrograph (Figure 10) at low magnification, x 11,000. High magnification, x 55,000.

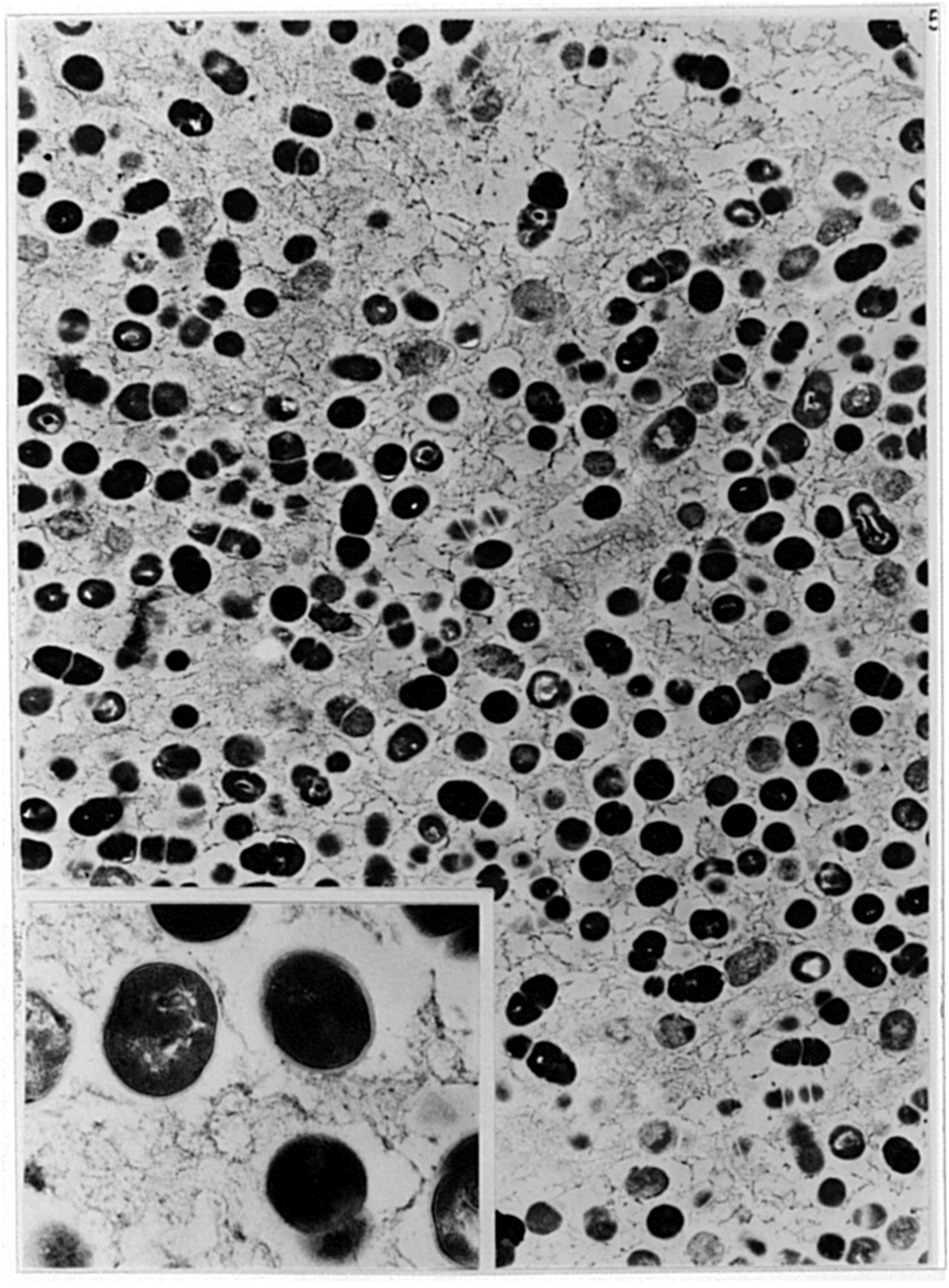

Figure 12: T.E.M. of S. mutans incubated for 3 days in Jordan's medium containing 5% sucrose and TiF_4 ($\simeq$ 2.3 ppm F). Low magnification, x 11,000, and high magnification, x 55,000, appear similar to the control photomicrograph (Figure 10).

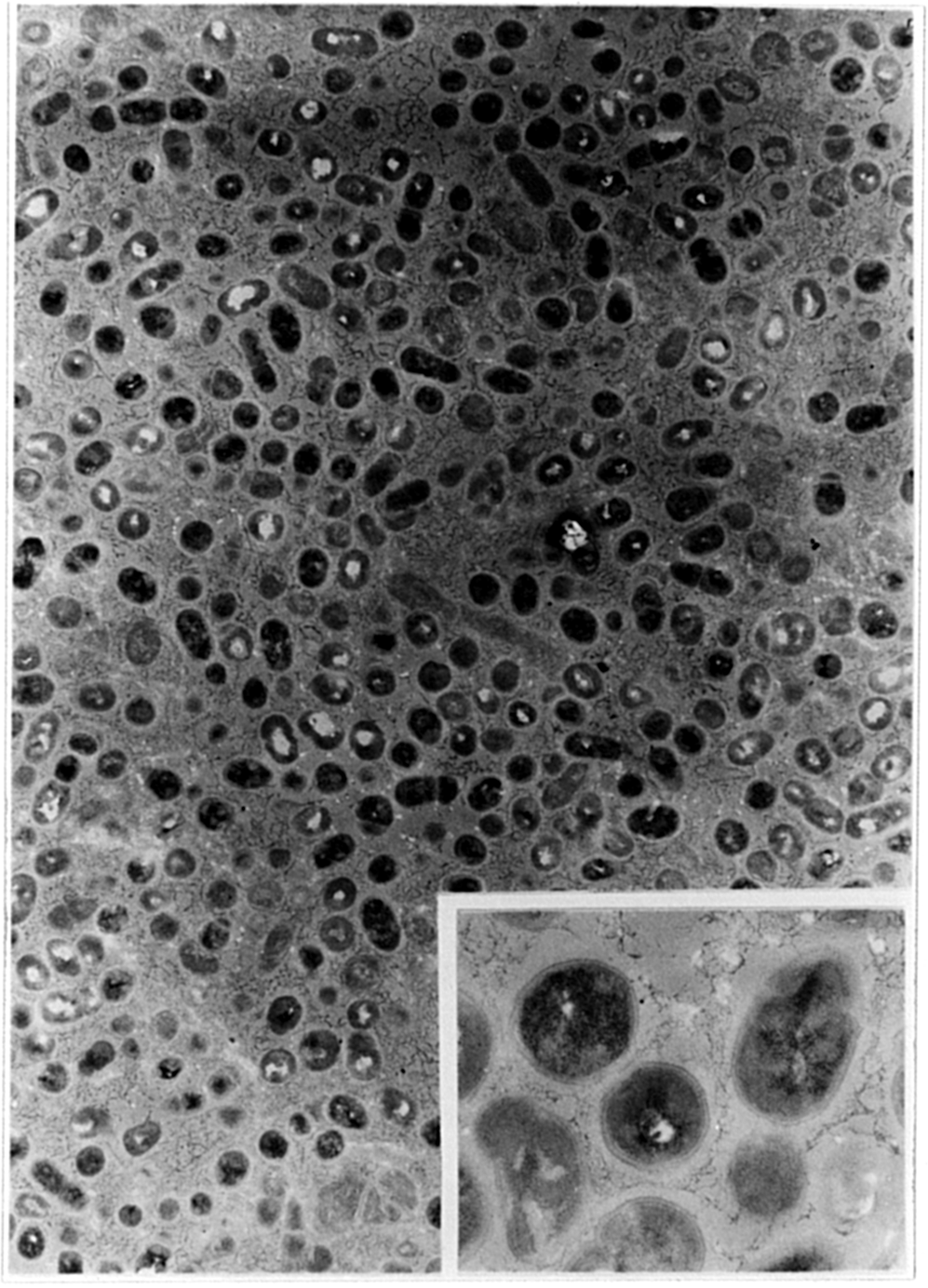

Figure 13: T.E.M. of S. mutans incubated for 3 days in Jordan's medium containing 5% sucrose and Na_2SnF_6 (10 ppm F). More extracellular material is apparent than in the control photomicrograph (Figure 10) at low magnification, x 11,000. High magnification, x 55,000.

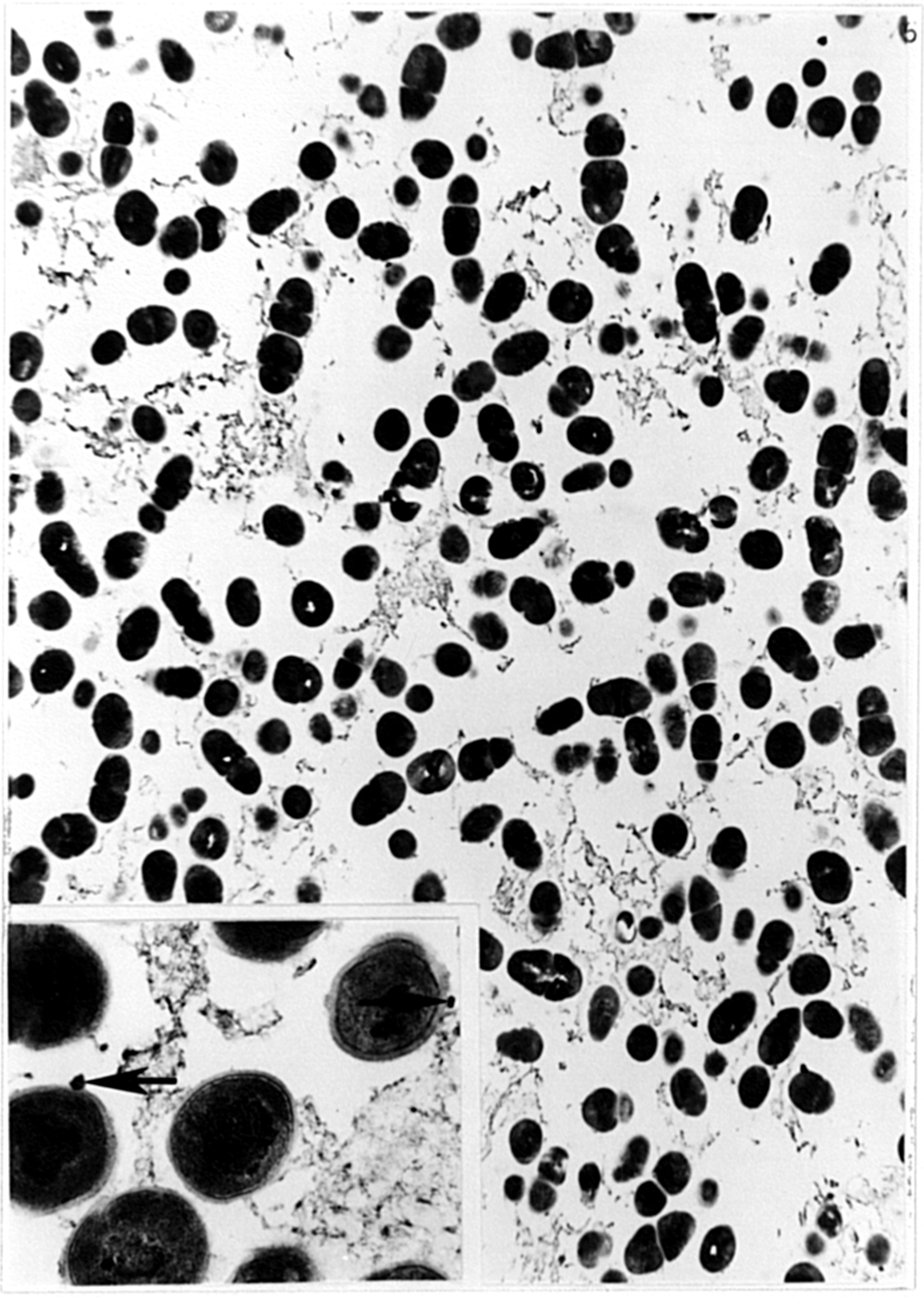

Figure 14: T.E.M. of S. mutans incubated for 3 days in Jordan's medium containing 5% sucrose and $SnCl_2$ (10 ppm Cl). Extracellular material at low magnification (x 11,000) appears to be less associated with the bacteria when compared to the control photomicrograph. High magnification (x 55,000) reveals electron dense granules most often associated with the bacterial cell wall (arrows). One electron dense granule can be noted within the cell.

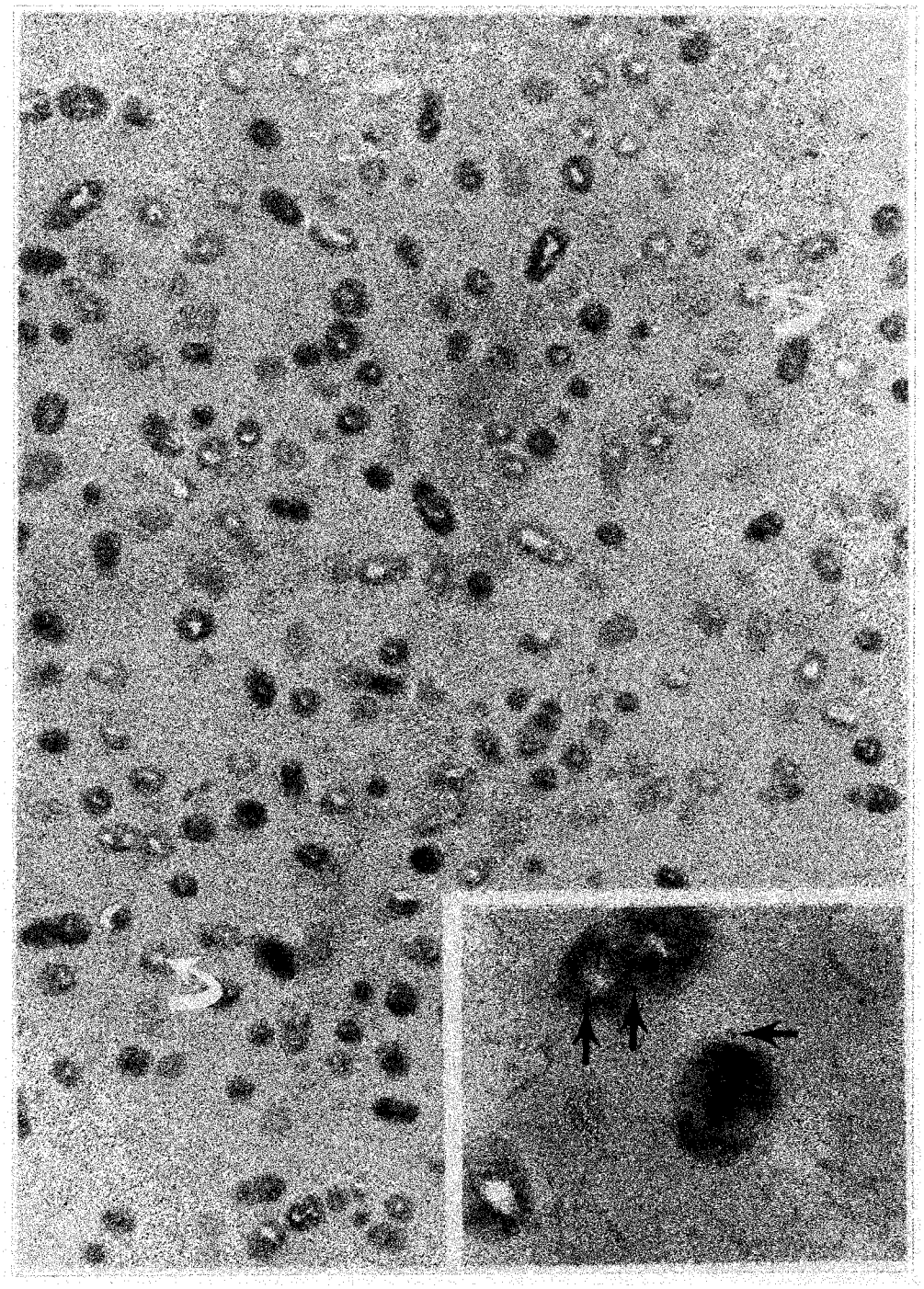

Figure 15: T.E.M. of S. mutans incubated for 3 days in Jordan's medium containing 5% sucrose and SnF_2 (10 ppm F). Note the presence of numerous electron lucent holes (white arrows) and apparently distorted cell shapes at low magnification, x 11,000. High magnification inset shows electron dense granules in the cell walls (black arrows), x 55,000.

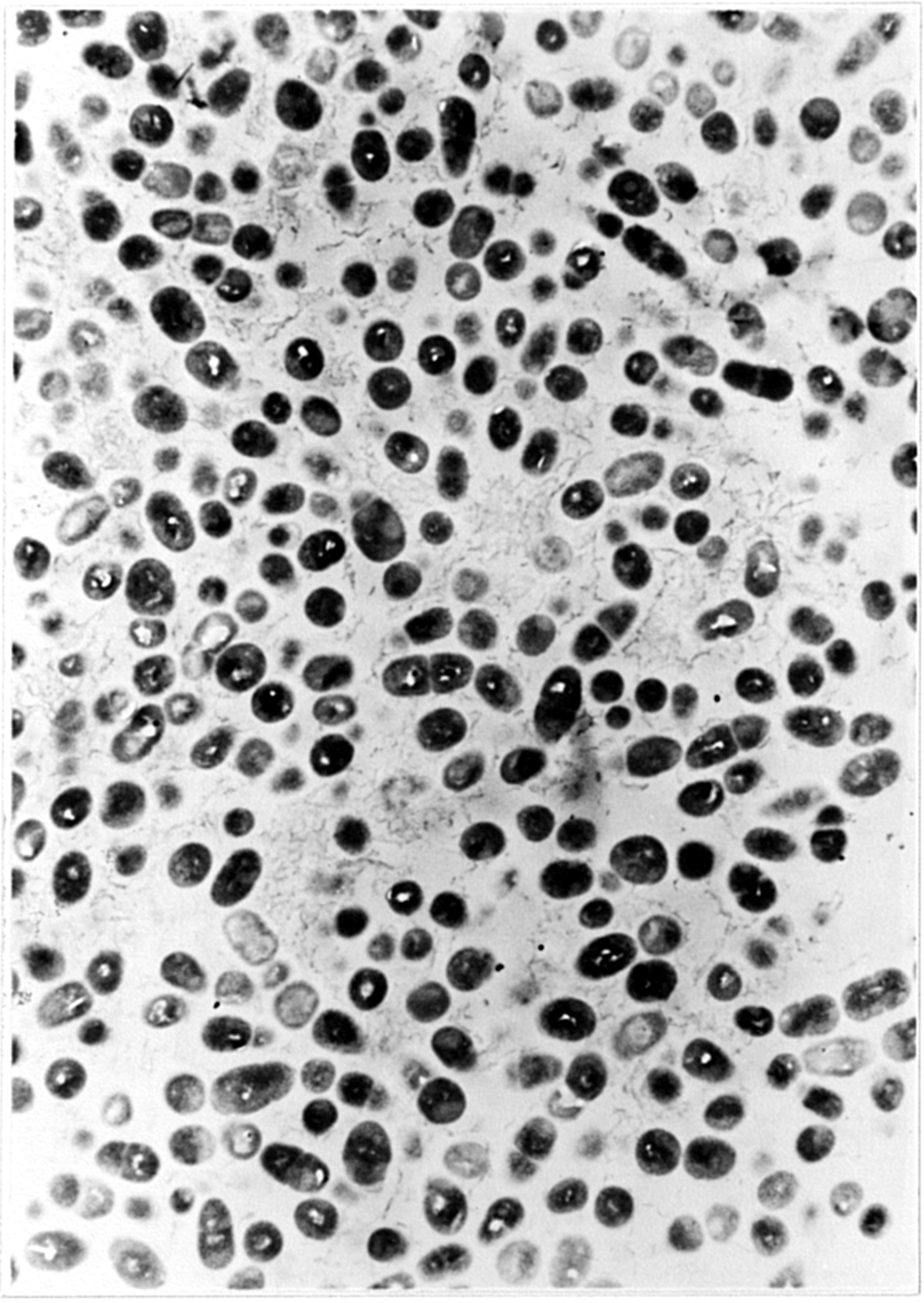

Figure 16: Electron micrograph of S. mutans incubated in medium containing $SnCl_2$ as in Figure 14 which was not stained with uranyl acetate and lead citrate so that semiquantitation of electron dense granules and electron lucent holes could be performed, x 11,000.

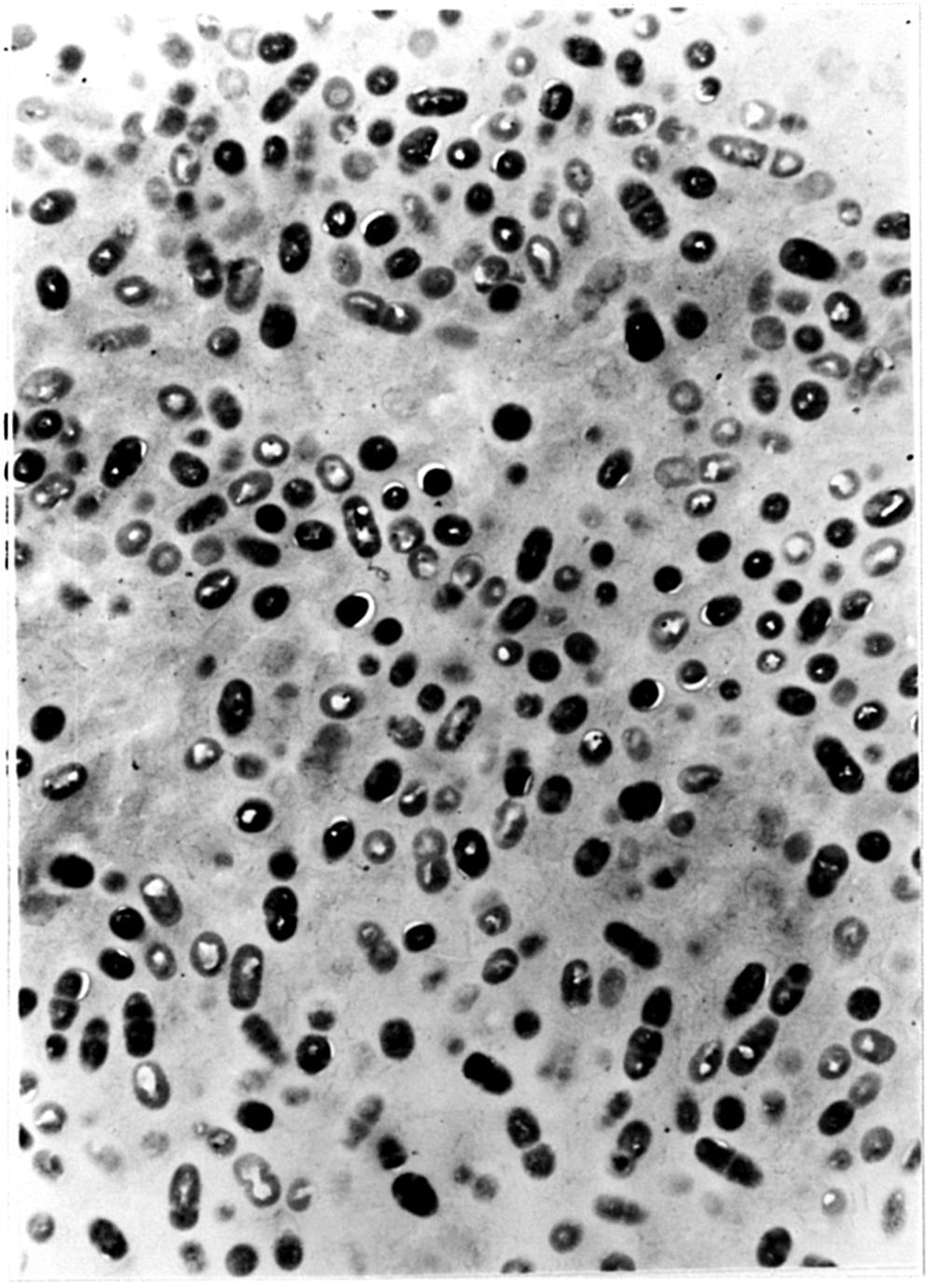

Figure 17: Electron micrograph of S. mutans incubated in medium containing SnF_2 as in Figure 15 which was not stained with uranyl acetate and lead citrate so that semiquantitation of electron dense granules and electron lucent holes could be performed, x 11,000.

Discussion

Electrode measurements of fluoride confirmed that the Jordan's medium, water, and $SnCl_2$ controls were essentially free of fluoride ion. Also, NaF, SnF_2, and Na_2SnF_6 had initial fluoride levels as expected from calculation. However, after 24 hours incubation, fluoride levels of these agents dropped approximately 1 ppm F in both inoculated and uninoculated media. This may indicate that some of the free fluoride present may become organically bound and therefore could not be detected. Unexpectedly, TiF_4 solutions did not give fluoride readings equal to the calculated levels, possibly because of the loss of HF as gas when this compound is added to water or because of the hydroscopic nature of this compound. The lower than expected fluoride levels of TiF_4 limited its value as a test agent in this study.

The MIC/MLC determinations were performed to determine the lowest concentration at which the various compounds have the ability to kill or completely inhibit growth of plaque forming bacteria. These were substantially greater that the 10 ppm F used in this study. SnF_2 exhibits the lowest fluoride and tin concentration at which both the minimum inhibitory and minimum lethal effect occurred. When the various test compounds were compared with respect to ppm F, SnF_2 had a MIC of 60 ppm and a MLC of 125 ppm. All other fluoride compounds exhibited a MIC of greater than 300 ppm and a MLC greater than 575 ppm. Confirmatory experiments were consistent for all compounds except TiF_4 perhaps because of the instability of this agent. With respect to the tin concentration of the compounds tested, SnF_2 had an MIC of 100 ppm while $SnCl_2$ and Na_2SnF_6 both had a MIC of 600 ppm and SnF_2 had a MLC of 375 ppm while $SnCl_2$ and Na_2SnF_6 had a MLC of 675 ppm. Since the lowest inhibitory concentration of the

agents tested was found to be 60 ppm F, the findings herein cannot be ascribed to the ability of these fluoride compounds to kill or completely inhibit the growth of S. mutans although even at 10 ppm F, some growth inhibition does occur.

NaF had a much wider discrepancy between values for MIC and MLC when compared to the other compounds tested, all of which contained heavy metals. The greater bactericidal effect of tin containing compounds is compatible with those effects noted for isolated heavy metals (Salle, 1968).

The relatively high bacteriostatic and bactericidal activity observed for SnF_2 at low concentrations cannot be explained by the separate action of tin alone or fluoride alone, since comparison of the results from SnF_2 to $SnCl_2$, NaF, or Na_2SnF_6 for either fluoride or tin effect clearly shows SnF_2 is more potent. The greater potency of this compound over NaF and $SnCl_2$ has been observed previously (Tinanoff et al., 1976c; Tinanoff and Camosci, 1980b). The mechanism for these differences may perhaps be explained by other aspects of this study.

The alteration of acid production by S. mutans seems to be due to the free fluoride ion released from the test agent rather than from the effect of the cation of the compound or from the combined effect of the fluoride ion with the cation. All of the fluoride agents tested except TiF_4 showed an effect on acid production at 10 ppm F and a lesser effect at 5 ppm F. Since TiF_4 had one-third the expected F, this fluoride agent was understandably less effective.

The effect of fluoride on bacterial metabolism has been known for some time and is well studied. Inhibition of acid production by salivary and plaque bacteria in vitro has been demonstrated with less than 1 ppm F (Bibby and van Kesteren, 1940; Wright and Jenkins, 1954). Furthermore,

plaque collected from subjects living in fluoridated areas exhibit less acid production on exposure to sucrose than plaque from subjects living in nonfluoridated areas (Jenkins et al., 1969). These findings may be explained by the observation that fluoride alters the bacterial enzyme, enolase, which is essential for the degradation of simple sugars to lactic acid and is also essential for the transport of sugars across the bacterial cell membrane (Hamilton, 1977; Slee and Tanzer, 1979). The inactivation of enolase is the result of fluoride binding with the magnesium component of this enzyme (Warburg and Christian, 1942). Fluoride ions acting in this manner could reduce bacterial acid production and might account for some of the caries inhibition noted for this agent.

Fluoride at 10 ppm was noted to alter the terminal pH of the test media by approximately 0.7 units. Since the critical pH needed for enamel dissolution is thought to be in the range of 4.5-5.5 (for review, see Fitzgerald, 1976), it is conceivable that this alteration in pH by fluoride may change the environment of the tooth from one fostering enamel demineralization to remineralization.

The bacterial growth yield was lower in all media supplemented with either fluoride or tin compounds, except for TiF_4. This decrease may be due to a fluoride effect on growth for all fluoride compounds tested and a tin effect on growth for $SnCl_2$ and SnF_2. SnF_2 had the greatest decrease in growth which may be due to a combined tin-fluoride effect.

Miller (1974, 1976) and Kashket et al. (1977) examined how various concentrations of fluoride alter both acid production and glucose uptake by S. faecalis and S. mutans. Results show a decrease in bacterial acid production when these bacteria were exposed to as little as 0.5 ppm F. However, there was no inhibition of glucose transport across the cell

membrane until at least 10 ppm F was used. In the present experiments, the adverse effect of the agents tested on growth at 10 ppm F may thus be due to the decreased metabolic activity of S. mutans as a result of decreased carbohydrate uptake.

Heavy metals, such as tin, are known to have a "germicidal" effect because of their ability to precipitate cellular proteins (Salle, 1968). Therefore, tin itself, may be metabolically disruptive, accounting for the decreased growth yield at a concentration of 10 ppm. The apparent combined tin-fluoride effect on growth of S. mutans at low concentrations of SnF_2 has been suggested by Tinanoff and Camosci (1980) to be the result of tin entering the cell passively coupled with fluoride. This could be one reason for the increased antiplaque effects of SnF_2 at low concentrations.

The DNA and glucan experiments examined the bacteria attached to the enamel after three days' growth and the unattached bacteria present in the culture media after the third day's growth. It should be noted that since the bacteria attached to the stainless steel wires were used for tin analyses, the DNA of these bacteria are not included in the total DNA calculations even though these bacteria may have contributed to the calculated total water soluble glucan. Hence, the total WSG/DNA ratio may not be accurate, and it is more appropriate to discuss WSG quantities as WSG/ml culture media rather than WSG/DNA.

Most studies that have evaluated the effect of fluoride on bacterial extracellular polysaccharide (EPS) production have observed decreases in EPS synthesis by bacteria grown under the influence of fluoride concentrations ranging from 10 to 70 ppm F (Loesche et al., 1973 and 1975; Bowen and Hewitt, 1974). Recently, Treasure and Handelman (1980) verbally reported EPS/bacterial protein data of several strains of S. mutans

incubated under the influence of 25 or 50 ppm F. In contrast to the earlier studies, they found increased EPS synthesis under the influence of fluoride. The results of the present study concur with those of Treasure and Handleman. The data herein, however, have the added benefit of measuring all bacterial cell DNA, not just viable cell mass and again show increase in EPS on a cellular basis.

With regard to the amount of alkali soluble glucan associated with the bacteria attached to enamel, this study does not find any differences in the amount of ASG/DNA between test groups or controls although there was a decrease in overall bacterial material present on enamel cultured in broth containing Na_2SnF_6, NaF, or SnF_2. However, when the total bacterial (unattached after the third day's growth plus attached to enamel) ASG/DNA ratios and ASG/ml culture media (unattached bacteria) ratios were assessed, an increase in ASG by the unattached bacteria in medium supplemented with SnF_2, Na_2SnF_6 and NaF ($p \leq .01$) can be noted. This increase in ASG could be due to either: (1) increased ASG production in the culture tube by the bacteria present not attached to the enamel specimens, or (2) the ASG produced by the bacteria attached to the enamel did not remain associated with these attached bacteria. Since enamel specimens were transferred daily into fresh uninoculated media, it is interesting to speculate that those bacteria that were not able to maintain attachment to the enamel specimens had increased ASG production. This hypothesis is supported by the fact that there was very little unattached bacterial mass present in the control tubes but was clearly present when fluoride compounds were present.

An overall increase in total water soluble glucan (WSG/DNA) for those test groups exposed to SnF_2, NaF, and Na_2SnF_6, was also observed. Since this extracellular polysaccharide is not cell associated, it is

not possible to determine if increased WSG production is primarily from the unattached or attached bacteria.

The disproportionately high levels of tin found by atomic absorption in those plaques treated with SnF_2 may be explained by several hypotheses. Rölla (1976) and Svatun et al. (1977) have suggested that tin ions may compete with calcium for acidic groups on the bacterial surface, thus allowing accumulation of this cation on the cell surface. Only indirect evidence exists to suggest the possible reason for the increased tin uptake by cells treated with SnF_2 over $SnCl_2$. Fluoride has been previously noted to be accumulated in plaque (Jenkins and Edgar, 1969), whereas chloride apparently is not concentrated by bacteria (Mitchell and Moyle, 1959; Schultz et al., 1962). Therefore, an increased accumulation of tin in bacterial cells from plaque exposed to SnF_2 could result from the passive entrance of tin coupled to fluoride (Tinanoff and Camosci, 1980a). Recently, Tinanoff and Camosci (1980b) have observed tin accumulation in or on cells exposed to concentrations of SnF_2 as low as 5 ppm F but not with $SnCl_2$ or Na_2SnF_6. These findings were confirmed in the present study. There was increased tin uptake with SnF_2 exposure compared to that of $SnCl_2$ or Na_2SnF_6. It should also be noted that whereas $SnCl_2$ and SnF_2 were adjusted for equimolar Sn concentrations, SnF_2 and Na_2SnF_6 were adjusted for equimolar F concentrations, not Sn concentrations. This may, in part, account for the decreased tin uptake by bacteria exposed to Na_2SnF_6 compared to the SnF_2 test group.

Further information on the mechanism by which the test agents effect S. mutans may be derived from the electron microscopic findings in the present study. The increase in extracellular material observed in electron micrographs of bacteria exposed to all fluoride test agents, except TiF_4, when compared to those of $SnCl_2$ or the H_2O control, is consistent with the glucan experiment results.

The electron lucent holes noted in the bacteria were a common finding in all of the test group photomicrographs. Such holds are compatible with the artifact which is seen when bacterial polyphosphate is examined under the electron microscope (Voelz et al., 1966). Polyphosphates have been identified in a variety of microorganisms (Harold, 1966) including S. mutans (Tanzer and Krichevsky, 1966; Tinanoff and Tanzer, 1979; Tinanoff and Camosci, 1980a). This highly anionic phosphate is believed found in cells when nutritional conditions are not favorable to growth (Harold, 1966).

The large number of holes found in bacteria exposed to $SnCl_2$ and especially SnF_2 may indicate unbalanced growth (Tinanoff and Camosci, 1980a). This finding confirms the altered growth patterns of those bacteria in the presence of Sn noted in other aspects of this study.

Electron dense granules observed in bacterial cells and on cell walls observed in electronmicrographs of unstained sections of S. mutans exposed to $SnCl_2$ or SnF_2 have been previously identified as tin deposits (Tinanoff and Camosci, 1980a, 1980b). The presence of twice as many tin granules intracellularly in the micrograph of S. mutans exposed to SnF_2 compared to those exposed to $SnCl_2$ is compatible with a hypothesis that tin enters the cell coupled to fluoride (Tinanoff and Camosci, 1980a). This semiquantitative finding appears to correlate well with this study's atomic absorption experiments in which bacteria exposed to $SnCl_2$ had 20 μg Sn/mg plaque and bacteria exposed to SnF_2 had 34 μg Sn/mg plaque. The tin accumulation may be the most important antiplaque determinant of SnF_2.

Summary and Conclusions

Previous literature has suggested that fluoride affects not only enamel solubility, as originally thought, but also bacterial growth and attachment. Unfortunately, most of these studies used only NaF, and very few reports have compared these antimicrobial effects with those of different fluoride compounds. In those studies that have compared various fluoride compounds, SnF_2 appeared to be the most effective antibacterial agent. Furthermore, most of the studies showing antibacterial effects of fluoride have been performed at concentrations which could be either bactericidal or bacteriostatic.

The effect of low levels of fluoride on bacterial plaque formation was studied using a variety of fluoride compounds added to growth medium inoculated with *S. mutans* NCTC 10449S. After establishing, through MIC and MLC testing, that 10 ppm F of the test agents was well below the MIC and MLC levels, the effect of these agents on bacterial growth, acid production, extracellular polysaccharide production, tin uptake, and attachment to enamel was investigated. The results of this series of studies indicate:

1. MIC and MLC values of SnF_2 and the other compounds tested do not account entirely for the effectiveness of these compounds at much lower concentrations.
2. *S. mutans* has decreased growth yield and decreased acid production when exposed to all test agents.
3. Although there was a decrease in total bacterial mass (attached to enamel and unattached) in the test groups continuously exposed to 10 ppm F, antiplaque mechanisms other than bacterial growth inhibition by these agents were suggested.

4. The increased effectiveness of SnF_2 over F as NaF or Sn as $SnCl_2$, as seen in the MIC, MLC, growth curves and DNA results, suggest a tin-fluoride interaction.
5. The large accumulation of tin found by atomic absorption spectrophotometry and electron microscopy in those organisms treated with SnF_2 may account for the increased effectiveness of SnF_2.
6. Tin uptake by bacteria exposed to $SnCl_2$ may partially explain the bacteriostatic effects of this compound.
7. Even though fluorides, especially SnF_2, affect the number of bacteria attached to enamel (μg DNA), these attached bacteria show no change in glucan production (ASG) as a result of exposure to the test agents during growth.
8. Fluoride increases extracellular polymer production by S. mutans as noted by electron microscopy and specifically increases alkali soluble glucan and water soluble glucan as noted by glucan analysis and DNA analysis.
9. Na_2SnF_6, NaF and especially SnF_2 appear to effect the attachment mechanisms of S. mutans during continuous exposure to 10 ppm F in the growth medium.
10. Bacteria exposed to tin, especially SnF_2, have an increase in electron lucent holes (bacterial polyphosphates) which may indicate unbalanced growth.
11. SnF_2 appears to be the most effective of those fluoride compounds treated when present at a concentration of 10 ppm F during growth of S. mutans NCTC 10449S.

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